JC10 Rec'd PCT/PTO 15 MAR 2002

Certificate of Mailing

Date of Deposit March 15, 2002 Label Number: EL954298065US

I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office to Addressee"** with sufficient postage on the date indicated above and is addressed to BOX PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.

Guy Beardsley
Printed name of person mailing correspondence

Guy Beardsley
Signature of person mailing correspondence

Substitute Form PTO 1390 U.S. Department of Commerce Patent and Trademark Office TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	Attorney's Docket Number: 50125/051001 U.S. Application Number: 10/088248

INTERNATIONAL APPLICATION NUMBER	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08996	September 14, 2000	September 15, 1999

TITLE OF INVENTION:	PHARMACEUTICAL COMPOSITION IN THE FORM OF A NUCLEIC ACID/LIPID COMPLEX, ITS PRODUCTION AND USE IN GENE THERAPY
APPLICANTS FOR DO/EO/US:	Jens Schletter

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1.	■ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2.	□ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3.	■ This is an express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4.	■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5.	A copy of the International Application as filed (35 U.S.C. § 371(c)(2)). □ a. is transmitted herewith (required only if not transmitted by the International Bureau). ■ b. has been transmitted by the International Bureau. □ c. Is not required, as the application was filed with the United States Receiving Office (RO/US).
6.	■ A translation of the International Application into English (35 U.S.C. § 371(c)(2).
7.	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)). □ a. are transmitted herewith (required only if not transmitted by the International Bureau). □ b. have been transmitted by the International Bureau. □ c. have not been made; however, the time limit for making such amendments has NOT expired. ■ d. have not been made and will not be made.
8.	□ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9.	■ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)). (Unsigned)
10.	□ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371 (c)(5).
11.	□ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98.
12.	□ An assignment for recording. A separate cover sheet in compliance with 37 §§ 3.28 and 3.31 is included.
13.	■ A FIRST preliminary amendment. □ A SECOND or SUBSEQUENT preliminary amendment.
14.	□ A substitute specification.
15.	□ A change of power of attorney and/or address letter.
16.	□ Other items or information:

17.	■ The following fees are submitted: BASIC NATIONAL FEE (37 C.F.R. § 1.492(A)(1)-(5)): Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.455(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $ 1040.00 International preliminary examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $ 890.00 International preliminary examination fee (37 C.F.R. § 1.482) not paid to USPTO but international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO $740.00 International preliminary examination fee (37 C.F.R. § 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1) - (4) $ 710.00 International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $ 100.00			$890.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				$890.00	
Surcharge of $130 for furnishing the oath or declaration later than □ 20 OR □ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	49-20=	29	x $18	$522.00	
Independent claims	7-3 =	4	x $84	$336.00	
Multiple dependent claims (if applicable)			+ $280	$280.00	
TOTAL OF ABOVE CALCULATIONS =				$2028.00	
Reduction of 1/2 for filing by small entity, if applicable. Applicant claims small entity status under 37 C.F.R. § 1.27.					
SUBTOTAL =				$2028.00	
Processing fee of $130.00 for furnishing the English translation later than □ 20 OR □ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)). +					
TOTAL NATIONAL FEE =				$2028.00	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). $40.00 per property. +					
TOTAL FEES ENCLOSED =				$2028.00	
				Amount to be refunded	$
				charged	$

■ a. A check in the amount of $2028.00 to cover the above fees is enclosed.
□ b. Please charge my Deposit Account No. 03-2095 in the amount of $ [**.**] to cover the above fees.
■ c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-2095.

NOTE: Where an appropriate time limit under 37 C.F.R. §§ 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:	
Karen L. Elbing, Ph.D. Clark & Elbing LLP 101 Federal Street Boston, MA 02110-2214 Telephone: 617-428-0200 Facsimile: 617-428-7045 Customer No.: 21559	Susan M. Michaud Signature Karen L. Elbing, Ph.D. Reg No. 35,238 Susan M. Michaud Reg. No. 42,885

\\Clark-w2k1\documents\50125\50125.051001 National Phase Transmittal.wpd

\\Clark-w2k1\documents\50125\50125.051001 National Phase Transmittal.wpd

21559
PATENT TRADEMARK OFFICE

10/088248
JC13 Rec'd PCT/PTO 15 MAR 2002

PATENT
ATTORNEY DOCKET NO. 50125/051001

Certificate of Mailing

Date of Deposit March 15, 2002 Label Number: EL954298065US

I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office to Addressee"** with sufficient postage on the date indicated above and is addressed to BOX PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.

Guy Beardsley
Printed name of person mailing correspondence

Guy Beardsley
Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Jens Schletter
Serial No.: Not yet assigned
Filed: March 15, 2002
Title: PHARMACEUTICAL COMPOSITION IN THE FORM OF A NUCLEIC ACID/LIPID COMPLEX, ITS PRODUCTION AND USE IN GENE THERAPY

Art Unit:
Examiner:
Customer No.: 21559

Assistant Commissioner For Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Prior to examination, kindly amend the above-referenced application as follows.

In the claims:

Please replace original claims 1-27 with the following new claims 28-76.

28. A pharmaceutical composition in the form of a nucleic acid/lipid complex, comprising:

(a) at least one cationic lipid (CL);

(b) at least one non-cationic lipid (NCL);

(c) at least one nucleic acid (N) coding for a protein for the treatment of vascular disorders; and

(d) wherein appropriate, suitable excipients and/or additives;

wherein the cationic lipid (CL) comprises a group which is derived from cholesterol and to which at least one cationic amino group selected from the group consisting of primary, secondary, tertiary amino group and a quaternary ammonium salt is linked via a connecting group selected from the group consisting of carboxamides and carbamoyls, and a spacer consisting of a linear or branched alkyl group having 1 to 20 carbon atoms, and wherein the size of the nucleic acid/lipid complexes is in a range of about 300-800 nm.

29. A pharmaceutical composition as claimed in claim 28, wherein the protein for the treatment of vascular disorders is a protein with vasodilating and/or angiogenic properties.

30. A pharmaceutical composition as claimed in claim 28, wherein the size of the nucleic acid/lipid complexes is in a range of about 350-550 nm.

31. A pharmaceutical composition as claimed in claim 28, wherein the nucleic acid (N) codes for one of the isoforms selected from the group consisting of nitric oxide synthase (NOS), hemoxygenase (HO), monocyte chemoattractant protein (MCP), and a variant of one of said proteins.

32. A pharmaceutical composition as claimed in claim 28, wherein the nucleic acid codes for a protein selected from the group consisting of inducible nitric oxide synthase

(iNOS), hemoxygenase-1 (HO-1), monocyte chemoattractant protein-1 (MCP-1), and a variant thereof.

33. A pharmaceutical composition as claimed in claim 32, wherein the nucleic acid codes for the human form of a protein.

34. A pharmaceutical composition as claimed in claim 28, wherein the cationic lipid (CL) is selected from the group consisting of 3β-[N-(N,N'-dimethylaminoethane)carbamoyl]cholesterol (DAC-Chol) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

35. A pharmaceutical composition as claimed in claim 28, wherein the non-cationic lipid (NCL) is at least one lipid selected from group consisting of phosphatidylcholine, phosphatidylethanolamine, and cholesterol.

36. A pharmaceutical composition as claimed in claim 35, wherein the phosphatidylethanolamine is a diacylphosphatidylethanolamine with a chain length of 10-28 carbon atoms.

37. A pharmaceutical composition as claimed in claim 36, wherein the phosphatidylethanolamine is selected from the group consisting of dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), and dioleylphosphatidylethanolamine (DOPE).

38. A pharmaceutical composition as claimed in claim 36, wherein the cationic lipid (CL) is DAC-Chol and the non-cationic lipid (NCL) is DOPE.

39. A pharmaceutical composition as claimed in claim 38, wherein the ratio of DAC-Chol to DOPE by weight is from about 10:90 to about 90:10.

40. A pharmaceutical composition as claimed in claim 38, wherein the ratio of DAC-Chol to DOPE by weight is about 30:70.

41. A pharmaceutical composition as claimed in claim 28, wherein the composition has been obtained by using total lipid composed of (CL) and (NCL) to nucleic acid (N) in the ratio of from about 1:1 to about 10:1 based on weight.

42. A pharmaceutical composition as claimed in claim 28, wherein the composition has been obtained by using total lipid composed of (CL) and (NCL) to nucleic acid (N) in the ratio of from about 4:1 or about 5:1 based on weight.

43. A pharmaceutical composition as claimed in claim 28, wherein the composition is in the form of a solution or lyophilisate.

44. A pharmaceutical composition as claimed in claim 28, wherein the excipient is a stabilizing agent.

10088248 .072402

45. A pharmaceutical composition as claimed in claim 44, wherein the excipient is selected from the group consisting of at least one sugar, at least one polyhydric alcohol, and at least one inorganic salt.

46. A pharmaceutical composition as claimed in claim 28, wherein the additive is selected from the group consisting of at least one molecule specifically recognizing target cells and at least one molecule facilitating gene transfer into the target cells.

47. A method for the production of the pharmaceutical composition as claimed in claim 28, the method comprising the following steps:

(i) providing of a mixture of a cationic lipid (CL) as set forth in claim 28 and a non-cationic lipid (NCL) as set forth in claim 28, and providing a nucleic acid (N) as set forth in claim 28;

(ii) mixing the mixture of (CL) and (NCL) with the nucleic acid (N);

(iii) optionally lyophilizing; and

(iv) optionally reconstituting.

48. A method as claimed in claim 47, wherein step (ii) the total lipid composed of (CL) and (NCL) and the nucleic acid (N) are mixed in the ratio of from about 1:1 to about 10:1 based on weight.

49. A method as claimed in claim 47, wherein the ratio is about 4:1 or about 5:1 based on weight.

50. A method as claimed in claim 47, wherein in step (i) providing of the mixture of (CL) and (NCL) and/or providing of the nucleic acid (N) takes place with use of a stabilizing agent.

51. A method as claimed in claim 50, wherein the stabilizing agent is selected from the group consisting of at least one sugar, at least a polyhydric alcohol, and at least an inorganic salt.

52. A method as claimed in claim 50, wherein the stabilizing agent is used in the form of an isoosmotic aqueous solution.

53. A pharmaceutical composition obtainable by the method as claimed in claim 47.

54. A method of carrying out gene therapy, the method comprising using the pharmaceutical composition as claimed in claim 28 for producing a pharmaceutical, optionally as a combination therapy with pharmacologically active substances.

55. The method as claimed in claim 54, wherein the method is used for the treatment of a disease selected from the group consisting of vascular disorders, genetically related

disorders, and disorders which can be treated by gene transfer, including prevention thereof.

56. The method as claimed in claim 54, wherein the method is used for the treatment or prevention of peripheral and/or coronary vascular disorders.

57. The method as claimed in claim 56, wherein the vascular disorder is selected from the group consisting of stenosis of vessels including vessel transplants, restenosis after percutaneous transluminal angioplasty (PTA) of coronary and/or peripheral vessels, a disorder resulting from hypoperfusion of tissues, coronary heart disease, myocardial infarction, vascular arteriosclerosis, and a disorder which leads to rejection of vessel and organ transplants.

58. The method as claimed in claim 54, wherein the method is used for local somatic gene therapy.

59. The method as claimed in claim 58, wherein the gene therapy takes place with the use of a catheter.

60. The method as claimed in claim 59, wherein the catheter is an Infiltrator catheter.

10088248 .072402

61. The method as claimed in claim 58, wherein the pharmaceutical composition is administered in a total dose in a range of about 0.1 – 20 μg based on the total amount of nucleic acid per administration.

62. The method as claimed in claim 58, wherein the pharmaceutical composition is administered in a total dose in a range of about 0.5 – 10 μg based on the total amount of nucleic acid per administration.

63. The method as claimed in claim 58, wherein the pharmaceutical composition is administered in a total dose in a range of about 1 – 5 μg based on the total amount of nucleic acid per administration.

64. A method of carrying out gene therapy, the method comprising using the pharmaceutical composition as claimed in claim 53 for producing a pharmaceutical, optionally as a combination therapy with pharmacologically active substances.

65. The method as claimed in claim 64, wherein the method is used for the treatment of a disease selected from the group consisting of vascular disorders, genetically related disorders, and disorders which can be treated by gene transfer, including prevention thereof.

66. The method as claimed in claim 64, wherein the method is used for the treatment or prevention of peripheral and/or coronary vascular disorders.

67. The method as claimed in claim 66, wherein the vascular disorder is selected from the group consisting of stenosis of vessels including vessel transplants, restenosis after percutaneous transluminal angioplasty (PTA) of coronary and/or peripheral vessels, a disorder resulting from hypoperfusion of tissues, coronary heart disease, myocardial infarction, vascular arteriosclerosis, and a disorder which leads to rejection of vessel and organ transplants.

68. The method as claimed in claim 64, wherein the method is used for local somatic gene therapy.

69. The method as claimed in claim 68, wherein the gene therapy takes place with the use of a catheter.

70. The method as claimed in claim 69, wherein the catheter is an Infiltrator catheter.

71. The method as claimed in claim 68, wherein the pharmaceutical composition is administered in a total dose in a range of about 0.1 – 20 μg based on the total amount of nucleic acid per administration.

72. The method as claimed in claim 68, wherein the pharmaceutical composition is administered in a total dose in a range of about 0.5 – 10 μg based on the total amount of nucleic acid per administration.

73. The method as claimed in claim 68, wherein the pharmaceutical composition is administered in a total dose in a range of about 1 – 5 μg based on the total amount of nucleic acid per administration.

74. A method for stabilizing nucleic acid/lipid complexes in solution, the method comprising using an isoosmotic composition comprising at least one component selected from the group consisting of a monosaccharide, a disaccharide, a polyhydric alcohol, and an inorganic salt.

75. A method for stabilizing nucleic acid/lipid complexes during lyophilization and/or reconstitution, the method comprising using an isoosmotic composition comprising at least one component selected from the group consisting of a monosaccharide, a disaccharide, a polyhydric alcohol, and an inorganic salt.

76. The method as claimed in claim 74 or 75, wherein the disaccharide is sucrose and the inorganic salt is sodium chloride.

REMARKS

By this amendment, Applicants have replaced the original set of claims with new claims 28-76, all of which find support in the original claim set. No new matter has been added.

If there are any other charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: March 15, 2002

Susan M. Michaud
Karen L. Elbing, Ph.D. Susan M. Michaud
Reg. No. 35,238 Reg. No. 42,885

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110-2214
Telephone: 617-428-0200
Facsimile: 617-428-7045

21559
PATENT TRADEMARK OFFICE

Pharmaceutical composition in the form of a nucleic acid/lipid complex, its production and use in gene therapy

The present invention relates to a pharmaceutical composition in the form of a nucleic acid/lipid complex comprising at least one cationic lipid, at least one non-cationic lipid, at least one nucleic acid coding for a protein for the treatment of vascular disorders, in particular a protein with vasodilating and/or angiogenic properties, and, where appropriate, suitable excipients and/or additives, where the cationic lipid (CL) comprises a group which is derived from cholesterol and to which at least one cationic amino group selected from primary, secondary, tertiary amino group and/or a quaternary ammonium salt is linked via a connecting group selected from carboxamides and carbamoyls, and a spacer consisting of a linear or branched alkyl group having 1 to 20 carbon atoms, and where the size of the nucleic acid/lipid complexes is in a range of about 300-800 nm. The present invention further relates to the production of the pharmaceutical composition and its use in gene therapy.

A number of pharmaceutical compositions in the form of a nucleic acid/lipid complex which comprise a cationic lipid and a nucleic acid and, where appropriate, further excipients and/or additives have already been developed.

Reszka (WO 96/20208 and DE 196 23 916) developed the cationic cholesterol derivative 3β-[N-(N,N'-dimethylaminoethane)carbamoyl]cholesterol (DAC-Chol) and described its use for direct liposomal gene transfer in vivo. In the production of the described liposomes, DAC-Chol was used with dioleylphosphatidylethanolamine (DOPE) as helper lipid in a molar ratio of 3:2. Epand, R.M. et al.

(US 5,283,185) described a method for transferring nucleic acids into cells using cationic lipids with protein kinase C-inhibitory properties, in particular the cationic lipid 3β-[N-(N',N'-dimethylamino-ethane)carbamoyl]cholesterol (DC-Chol) and a co-lipid such as DOPE. An acid addition salt of DC-Chol and its use for transfection of animal cells by means of a liposomal system has likewise been described (US 5,753,262). In addition, amphipatic vehicles comprising polyamine/cholesterol conjugates have been proposed for transferring nucleic acids into cells, e.g. for gene therapy (US 5,614,503). A liposomal condition which comprises a cationic lipid together with a neutral co-lipid, where the liposomes have a size of about 800 nm, has been described for transferring a DNA molecule (WO 98/17814). Complexes of nucleic acid and cationic liposomes have also been proposed for achieving an organ-specific gene expression in a mammal (US 5,676,954). Szoka, F.C. et al. (US 5,811,406) developed a transfection method using a lipid/polynucleotide complex which was stabilized by addition of a cryoprotective compound and was subsequently lyophilized. The lyophilized complexes were employed directly for the transfection, without reconstitution. Sorgi, F.L. and Huang, L. (WO 96/27393) described a dry powder formulation which comprises a lyophilized nucleic acid/liposome complex. The lyophilized complex can be employed after reconstitution as an aerosol for gene transfer in vitro and in vivo. In particular, DC-Chol and DOPE were employed as lipids, optionally with a sugar as cryoprotective compound. Bischoff, R. (WO 98/08489) described lipid/nucleic acid complexes which comprised a cationic lipid (e.g. DC-Chol), a co-lipid (e.g. DOPE), a stabilizing additive (PEG and derivatives thereof) and a nucleic acid. The resulting particles had a size of 500 nm or less. In addition, Marshall, J. et al. (WO 98/13026) proposed a composition which comprises a cationic amphiphilic

10088248 .072402

molecule, a nucleic acid molecule and preferably a co-lipid. Finally, Debs, R.J. and Zhu, N. (US 5,827,703) described a liposomal complex for systemic introduction of genetic material into a mammal, the complex comprising a cationic lipid in conjunction with cholesterol as non-cationic lipid.

One example of the production of DNA/liposome complexes from phosphatidylcholine, phosphatidylserine and cholesterol and the successful use thereof in the transfection of vessel walls with the aid of Sendai viruses has been described in DE 44 11 402. A transfection system comprising an Infiltrator catheter, a nucleic acid in the form of a nucleic acid/liposome complex and, where appropriate, suitable excipients and/or additives, and the use thereof for treating vascular disorders are disclosed in DE 197 29 769.

It is clearly evident from the aforementioned documents that numerous efforts have been made to provide a transfection system of maximal suitability for use in gene therapy. This also applies in particular to the gene therapeutic treatment of vascular disorders such as high blood pressure, arteriosclerosis, stenosis or restenosis.

It was an object of the work for the present invention to find a formulation and a method for the nonviral transfer of nucleic acids into vascular cells, which assists efficient transfer of the therapeutic gene into the target cells, brings about local accumulation of a therapeutic protein in the vessel wall with minimal damage to the surrounding tissue and enables a lyophilisate to be produced commercially in a form suitable for marketing.

It has now been found, surprisingly, that therapeutic liposomal formulations can be transferred with very

high efficiency locally into cells of the vessel wall when nucleic acids are formulated with suitable lipids and suitable additives and/or excipients to give complexes of a defined size, and these complexes are introduced into the target cells of the vessel wall.

One aspect of the present invention is therefore a pharmaceutical composition in the form of a nucleic acid/lipid complex comprising at least one cationic lipid, at least one non-cationic lipid, one nucleic acid coding for a protein for the treatment of vascular disorders, in particular a protein with vasodilating and/or angiogenic properties, and, where appropriate, suitable excipients and/or additives, where the cationic lipid (CL) comprises an, group which is derived from cholesterol in particular a lipophilic, and to which at least one cationic amino group selected from primary, secondary, tertiary amino group and/or a quaternary ammonium salt is linked via a connecting group selected from carboxamides and carbamoyls, and a spacer consisting of a linear or branched alkyl group having 1 to 20, preferably 1 to 10, carbon atoms, and where the size of the nucleic acid/lipid complexes is in a range of about 300-800 nm. Particularly good results can be achieved when nucleic acid/lipid complexes with a size of about 350-550 nm are employed for the gene transfer, in particular *in vivo*.

The nucleic acid/lipid complexes are normally liposomal complexes. These liposomal complexes, especially the nucleic acids, generally comprise no viral constituents. The nucleic acid is normally genomic DNA, cDNA, synthetic DNA, RNA, mRNA, ribozymes, antisense RNA, synthetic peptide-nucleic acids and oligonucleotides, preferably a cDNA. The nucleic acid can be used for example in the form of a suitable DNA expression vector (see, for example, DE 44 11 402). The protein or polypeptide employed for treating vascular

disorders is preferably one with vasodilating and/or angiogenic properties. Examples of proteins with vasodilating properties are the isoforms of nitric oxide synthase (NOS) and hemoxygenase (HO), while the isoforms of monocyte chemoattractant protein (MCP) have angiogenic properties. The family of nitric oxide synthases comprises at least three different isoenzymes: the endothelial enzyme (eNOS), the neuronal enzyme (nNOS) and the inducible NOS (iNOS) (see, for example, DE 44 11 402 and DE 197 29 769). The family of hemoxygenases likewise comprises at least three isoenzymes: hemoxygenase 1 (HO-1), hemoxygenase 2 (HO-2) and hemoxygenase 3 (HO-3) (see, for example, Soares, M.P. et al. (1998) Nature Medicine 4, 1073; Hancock, W.W. et al. (1998) Nature Medicine 4, 1392; Yoshida, T. et al. (1988) Eur. J. Biochem. 171, 457; McCoubrey, W.K. Jr et al. (1992) Arch. Biochem. Biophys. 295, 13; McCoubrey, W.K. Jr et al. (1997) Eur. J. Biochem. 247, 725). The isoforms of monocyte chemoattractant protein include, inter alia, the following proteins: MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5 (see, for example, US 5,212,073; US 5,278,287; Ito, W.D. et al. (1997) Circ. Res. 80, 829; Arras, M. (1998) J. Clin. Invest. 101, 40; WO 97/35982 and WO 98/44953). A particularly preferred nucleic acid is one which codes for inducible nitric oxide synthase (iNOS), hemoxygenase 1 (HO-1), monocyte chemoattractant protein-1 (MCP-1) or a variant thereof, with particular preference in turn for the human form in each case.

Besides its vasodilating action, iNOS has an antithrombotic and antiproliferative action. Mediators of inflammation such as endotoxins lead to increased expression of this enzyme, which is also referred to as inducible nitric oxide synthase (see also DE 44 11 402 and DE 197 29 769). The hemoxygenase isoforms HO-1 and HO-2 are expressed inter alia in the vascular system and break down heme into the bile pigment biliverdin.

10088248 .072402

This takes place with liberation of free iron and carbon monoxide (CO). The latter has, like the nitric oxide (NO) generated by NOS, a vasodilating and antithrombotic action. Although the CO produced by HO-1 inhibits, just like NO, the proliferation of smooth muscle cells, there is no induction by HO-1, in contrast to NOS, of apoptotic cell death. HO-1 has moreover been described as antiinflammatory and immunoprotective in transplantation models (Hancock et al., *supra*; and Soares et al., *supra*). Isoform 1 of monocyte chemoattractant protein (also called monocyte chemotactic protein-1 or JE cytokine), MCP-1, is important in arteriogenesis, i.e. formation of collateral vessels from arterioles which are already present. It is possible by introducing the MCP-1 protein into artificially induced occlusion points in the animal model of the rabbit femoral artery (Ito et al., *supra*; Arras et al., *supra*) to achieve the formation of collateral vessels which restore the blood flow into the treated extremities.

The present invention also encompasses variants of the aforementioned proteins. The term "variant" means herein proteins or polypeptides which have a sequence homology, in particular a sequence identity of about 70%, preferably of about 80%, in particular of about 90%, especially of about 95%, to the aforementioned proteins. These also include deletions of the protein in the region of about 1-60, preferably of about 1-30, in particular of about 1-15, especially of about 1-5, amino acids. These additionally include fusion proteins which comprise the aforementioned proteins. Variants also mean allelic variants which originate from other cells or tissues. Also meant thereby are proteins derived from different individuals. Accordingly, the present invention also encompasses nucleic acids which code for the aforementioned proteins or polypeptides. Examples of such related nucleic acids are nucleic

10088248 .072402

acids from different human cells or tissues or allelic variants, and nucleic acids which may be derived from different human individuals. In the wider sense, a "variant" of a nucleic acid of the present invention means a nucleic acid which has a homology, in particular a sequence identity of about 50%, preferably about 75%, in particular of about 90% and especially of about 95%. Suitable techniques and methods for the production and mutagenesis of nucleic acids and for gene expression and protein analysis are available to the skilled worker (see, for example, Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press; Glover, D.M. (1995) DNA cloning: A practical approach, Volume II: Expression systems, IRL Press; Ausubel et al. (1992) Short Protocols in Molecular Biology, John Wiley & Sons; Rees, A.R. et al. (1993) Protein Engineering: A practical approach, IRL Press). The term "at least one nucleic acid" means according to the present invention that the nucleic acid/lipid complexes may also comprise combinations of more than one nucleic acid, in which case the nucleic acids may code both for different forms of a therapeutic protein and for different therapeutic proteins among those described herein.

In a preferred embodiment, the cationic lipid is 3β-[N(N'N'-dimethylaminoethane)carbamoyl]cholesterol (see, for example, Epand et al., *supra*) or 3β-[N-(N,N'-dimethylaminoethane)carbamoyl]cholesterol (DAC-Chol) (see, for example, Reszka, *supra*).

The at least one non-cationic lipid is normally a lipid selected from at least one phosphatidylcholine, at least one phosphatidylethanolamine and/or cholesterol. The phosphatidylethanolamine is preferably a phosphatidylethanolamine with a chain length of 10-28 carbon atoms, in particular dimyristoylphosphatidylethanolamine (DMPE),

10088248 .072402

dipalmitoylphosphatidylethanolamine (DPPE) and/or dioleylphosphatidylethanolamine (DOPE), with particular preference DOPE.

A particularly advantageous pharmaceutical composition has proved to be one in which the cationic lipid is DAC-Chol and the non-cationic lipid is DOPE, preferably in a ratio of DAC-Chol to DOPE by weight of from about 10:90 to about 90:10, this also encompassing all ratios by weight which lie between these stated ratios, such as about 20:80, 30:70, 40:60, 50:50, 60:40, 70:30 and 80:20, but in particular also ratios in between these, such as, for example, 21:79, 22:78, 23:77, 24:76, 25:75, 26:74, 27:73, 28:72, 29:71 and 31:69, 32:68, 33:67, 34:66, 35:65, 36:64, 37:63, 38:62 and 39:61. A ratio of DAC-Chol to DOPE by weight of about 30:70 is particularly preferred. As an alternative to this it is also possible to express the ratio of the cationic lipid to the non-cationic lipid as molar ratio.

The composition of the invention is obtained with use of total lipid composed of cationic lipid and, where appropriate, non-cationic lipid to nucleic acid in the ratio of from about 1:1 to about 10:1, in each case based on weight. It is particularly advantageous to use a ratio by weight of from about 4:1 to about 5:1, with which a surprisingly good transfection efficiency is obtained *in vivo*.

The composition of the invention can be in the form of a solution, in particular of a freshly prepared aqueous solution, or of a lyophilisate, which can be employed after reconstitution.

The composition of the invention preferably comprises one or more excipients. An agent particularly preferred in this connection is one which stabilizes the nucleic acid/lipid complexes both in unlyophilized and

10088248.072402

lyophilized form. A measure which can be used for the stability of the complexes is the constancy of the size of these complexes with time. The stabilizing agent is advantageously at least one sugar, at least one inorganic salt and/or at least one polyhydric alcohol. In a wider sense, a sugar in this connection also means a sugar alcohol such as, for example, mannitol. An example of a polyhydric alcohol is polyethylene glycol (PEG). The combination of sucrose as sugar and NaCl as inorganic salt is particularly preferred. The method proposed hereinafter for producing the compositions of the invention provides, for example, for inducing this combination of substances by means of an isoosmotic solution in a suitable step of the method. Further possibilities for inclusion of the stabilizing agent will be evident to the skilled worker in the light of the present description.

The composition of the invention may additionally comprise at least one additive. This preferably takes the form of at least one molecule which specifically recognizes the target cells and/or one molecule which facilitates gene transfer into the cells. The specific recognition of cells is also referred to as "targeting". At least two possibilities are available in principle for this targeting: on the one hand it is possible to use antibodies against structures on the cell surface such as, for example, receptors, which are integrated into viral or liposomal vector systems (Vingerhoeds, H. et al. (1994) Immunmeth 4, 259; Wickham, T.J. et al. (1996) J. Virol. 70, 6831), and on the other hand it is possible to use peptides with high binding affinity for receptors on the cell surface. Accordingly, the term "one molecule which specifically recognizes the target cells" means in this connection cell- or tissue-specifically binding antibodies or peptides. Peptides of this type may be known as parts of a receptor-ligand system or can be isolated, for

10088248 .072402

example, by means of a screening of a combinatorial peptide library (Lu, Z. et al. (1995) Biotechnol. 13, 366; U.S. Patent No. 5,635,182; Koivunen, E. et al. (1999) J. Nucl. Med. 40, 883). Besides the peptides described, a number of other molecules likewise able to contribute to specific recognition of a target cell are conceivable. These include not only pharmacological active substances but also nucleic acid aptamers which are able to bind specifically structures on the cell surface (Hicke, B.J. et al. (1996) J. Clin. Invest. 98, 2688). Molecules which facilitate gene transfer into the cells may act in a variety of ways. They may, on the one hand, consist of proteins or peptides which are bound to a DNA or a synthetic peptide-nucleic acid and facilitate transport of the nucleic acid into the cell nucleus of the cell (Schwartz, B. et al. (1999) Gene Therapy 6, 282; Brandén, L.J. et al. (1999) Nature Biotechnology 17, 784). They may additionally be molecules which improve the release of the nucleic acid into the cytoplasm of the cell (Planck, C. et al. (1994) J. Biol. Chem. 169, 12918; Kichler, A. et al. (1997) Bioconjug. Chem. 8, 213), or molecules which improve the stability of the nucleic acid in the cell, such as the DNA-condensing cationic polymers poly-L-lysine and polyethyleneimine (Lechardeur, D. et al. (1999) Gene Therapy 6, 482).

A further aspect of the present invention is a method for producing the pharmaceutical composition of the invention, which comprises the following steps:

(i) provision of a mixture of at least one appropriate cationic lipid (CL) and at least one appropriate non-cationic lipid (NCL), and provision of at least one nucleic acid defined herein;

(ii) mixing of the mixture of (CL) and (NCL) with the at least one nucleic acid (N);

10088248 .072402

(iii) where appropriate lyophilization; and

(iv) where appropriate reconstitution.

The "provision" of said starting materials means herein both the preceding production of a starting material and the use of a previously produced, possibly commercially available, starting material. It may in the latter case be advisable to check the particular concentrations stated before use and, where appropriate, adjust appropriately.

In step (ii) normally the total lipid composed of (CL) and (NCL) and the nucleic acid (N) is mixed in the ratio of from about 1:1 to about 10:1, in each case based on weight. It is evident that all the ratios by weight between these values are also encompassed by the present disclosure, e.g. about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, in particular also all ratios by weight which increase by the value 0.1 in each case, e.g. 4.1:1, 4.2:1, etc. Ratios by weight of from about 4:1 to about 5:1 are particularly preferred, because a surprisingly high transfection efficiency, especially *in vivo*, can be found with these ratios.

In a preferred embodiment of the proposed method, in step (i) there is provision of the mixture of (CL) and (NCL) and/or provision of the nucleic acid (N) with use of a stabilizing agent, in particular of at least one sugar, of at least one inorganic salt and/or of at least one polyhydric alcohol. As stated above, this agent counteracts in particular the disadvantageous effects of any lyophilization. The stabilizing agent can be used in the form of an isoosmotic (about 300-330 mOsm) solution. A particularly preferred example of a stabilizing agent is a combination of sucrose as sugar and NaCl as inorganic salt. It is advantageous to employ this combination in the form of an isoosmotic aqueous solution, for example with a concentration of

100 mM sucrose/100 mM NaCl or 250 mM sucrose/25 mM NaCl. Nucleic acid/lipid complexes produced using the latter solutions have a particularly good stability and bring about efficient transfection *in vitro* and *in vivo*.

A further aspect of the present invention is therefore a pharmaceutical composition which can be obtained by the method proposed herein.

The present invention additionally relates to the use of the pharmaceutical composition of the invention in gene therapy, including a combination therapy with pharmaceutical active substances. It may in this connection be expedient to combine a gene therapy with other therapeutic approaches, such as, for example, administration of pharmacological active substances including proteins and/or peptides. For example, a gene therapy with iNOS can be combined with administration of nitrates, calcium channel blockers and/or β-adrenoreceptor antagonists (in relation to the latter administration, see Dieterich, H.A. et al. (editors) Koronare Herzkrankheit, WVG GmbH, Stuttgart, 1993). Preference is given to the treatment of vascular disorders, genetically related disorders and/or disorders which can be treated by gene transfer, including prevention thereof. Particular preference is given to the use thereof for the treatment and prevention of peripheral and/or coronary vascular disorders. Examples are, inter alia, high blood pressure, arteriosclerosis, including of the arteriosclerosis of transplants, and the stenosis or restenosis of vessels, including vessel transplants, in particular also of coronary heart disease and of myocardial infarction. Treatment/prevention of stenosis of vessels, including vessel transplants, of restenosis after a percutaneous transluminal angioplasty (PTA) of coronary and/or peripheral vessels, of hypoperfusion of

tissues (revascularization of the ischemic tissues is the aim in this case), of coronary heart disease, of myocardial infarction and/or of vascular arteriosclerosis, in particular after transplantation of vessels and/or organs, is in turn particularly preferred in this connection. A further preferred embodiment is the use of the pharmaceutical composition described herein for local somatic gene therapy. In this case, therapeutically active genes can be transferred locally via somatic gene transfer into the vessel wall and be expressed there, which makes it possible to prolong the therapeutic action compared with local administration of medicaments (see, for example, DE 197 29 769). Particular preference is given to local somatic gene therapy using a minimally invasive and efficient catheter technology with which targeted transfection of individual vessel sections *in vivo* is possible. An Infiltrator® catheter (see, for example, DE 197 29 769) is particularly preferred for this purpose.

The pharmaceutical composition will normally be administered with a total dose in a range from about 0.1 to about 20 μg (including all values in between), based on the total amount of nucleic acid. It is clear to the skilled worker in this connection that "value in between" means any value between the stated upper and lower limits, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, etc.; 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, etc.; 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, etc.; 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, etc.; 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, etc.; 5.0, etc.; 6.0, etc.; 7.0, etc.; 8.0, etc.; 9.0, etc.; 10.0, etc.; 11.0, etc.; 12.0, etc.; 13.0, etc.; 14.0, etc.; 15.0, etc.; 16.0, etc.; 17.0, etc.; 18.0, etc.; 19.0, etc.; 20.0. It is obvious that the dose actually employed, the exact composition, the time and the mode of administration, and the further details of the treatment can be varied in the light of the present

disclosure. A suitable animal model which can be employed is either the normal domestic pig (Schwartz, R.S. et al. (1990), Circulation 82, 2190; Karas, S.P. et al. (1992) J. Am. Coll. Cardiol. 20, 467) or the so-called minipig (Tumbleson, M.E. and Schook, L.B. (1996) Advances in swine in biomedical research, Plenum Press, New York, vol. 2, 684; see also Unterberg, C. et al. (1995) J. Am. Coll. Cardiol. 26, 1747). The results found in these models can then be applied appropriately to humans. The pharmaceutical composition is preferably administered with a total dose in a range from about 0.5 µg to about 10 µg, particularly preferably about 1 µg to about 5 µg, in each case based on the total amount of nucleic acid.

On use of iNOS as therapeutically active gene it is possible to achieve not only a vasodilating and antithrombotic action but also, in particular, an antiproliferative action on the smooth muscle cells of the vessel wall. In the aforementioned animal model, surprisingly, a better transfection efficiency *in vivo* was found with a total dose of 2 µg of DNA than with a total dose of 10 µg. On use of HO-1 as therapeutically active gene it is possible to achieve a vasodilating and antithrombotic action. In addition, HO-1 is described as being antiinflammatory and immunoprotective in transplantation models (Hancock et al. (1998), *supra*; and Soares et al. (1998), *supra*). Accordingly, HO-1 can be employed as therapeutically active gene in the pharmaceutical composition described herein also for antiinflammatory and immunoprotective effects on transplants. Introduction of an HO-1-expressing plasmid, for example into aortic transplants in the rat, is said to prevent the development of arteriosclerosis in the transplanted vessel sections. In a further step it is possible to prevent, through expression of HO-1, the development of arteriosclerosis in venous transplants, for which purpose the animal

10088248 .072402

models of mouse, rat, pig (see above) and monkey are likewise suitable. Gene transfer in this case can take place either by means of an Infiltrator® catheter into the vessel wall or by perfusion of the vessel transplants. A central area of clinical use is thus the prevention of arteriosclerosis in vessel and organ transplants. Useful treatment of coronary heart disease and myocardial infarction, and of coronary and peripheral occlusive diseases in analogy to NOS is also possible. On use of MCP-1 as therapeutically active gene in the pharmaceutical composition described herein it is possible to induce both arteriogenesis (i.e. the formation of collateral vessels from arterioles which are already present) and angiogenesis (i.e. the formation of new capillaries). Introduction of an MCP-1-expressing plasmid by means of an Infiltrator® catheter into peripheral or coronary vessels is intended to induce revascularization of ischemic tissues. It is possible to employ for this purpose the rabbit model described by Ito et al. (*supra*) (especially for peripheral vessels), and the animal models of the pig described herein (in particular for use on coronary vessels). The Infiltrator® catheter is intended for administration to both species. Areas of therapeutic use are thus, in particular, coronary heart disease, peripheral occlusive diseases and myocardial infarction. In a particular embodiment, MCP-1 is used together with GM-CSF. "Colony-stimulating factors" (CSFs) are proteins which mediate the proliferation and differentiation of hematopoietic precursor cells. The members of this protein family are named according to the cell types whose proliferation and differentiation they stimulate: M-CSF (also called CSF-1, and its alternative splice forms such as CSF-4) acts specifically on macrophages, G-CSF (CSF-3) on granulocytes, while GM-CSF (CSF-2) stimulates both cell types. A further member of this protein family is multi-CSF, which is known as interleukin-3. The cDNA

sequence of human GM-CSF has been described by Wong, G.G. et al. (Science 228, 810, 1985). The effect of GM-CSF in arteriogenesis, where appropriate acting synergistically with MCP-1, is activation and proliferation of macrophages, which results in the formation of collateral vessels from arterioles (WO 99/17798).

A further aspect of the present invention is therefore a method for the therapeutic and/or prophylactic treatment of a subject, where the method comprises the administration of an effective amount of the pharmaceutical composition described above.

Finally, a further aspect of the invention relates to the use of an isoosmotic composition comprising at least one mono- and/or disaccharide, and/or at least one polyhydric alcohol and/or at least one inorganic salt for stabilizing nucleic acid/lipid complexes in solution and/or during lyophilization and/or reconstitution. Particular preference is given on use of the composition for stabilizing nucleic acid/lipid complexes in solution to embodiments 1-5 detailed in the following table, and for stabilization during lyophilization and/or reconstitution to embodiments 1-6.

It is particularly advantageous to use for this purpose a composition which comprises sucrose as disaccharide and sodium chloride as inorganic salt. An example of such an isoosmotic composition (e.g. 300 mOsm) is a combination of sodium chloride in a concentration in a range from about 5 mM to about 100 mM, in particular 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 mM, with an appropriate amount of sucrose. In addition, use of a composition comprising mannitol alone or in combination with at least one other mono- and/or disaccharide such as, for example, sucrose or trehalose is preferred for

10088248 .072402

the aforementioned purposes. For example, such an isoosmotic composition (e.g. 300 mOsm) may comprise a combination of mannitol in a concentration in a range of about 10-290 mM, in particular about 150-290 mM, and sucrose or trehalose correspondingly in a concentration in a range of about 10-290 mM, in particular about 10-150 mM.

Embodiment	**M-/d-saccharide**	**Polyh. alcohol**	**Inorg. salt**
1	+	+	
2	+		+
3	+	+	+
4		+	
5		+	+
6			+

The following figures and examples are intended to explain the invention in detail without restricting it thereto.

10088249.072402

DESCRIPTION OF THE FIGURES

Fig. 1 shows the expression of iNOS *in vitro* in porcine smooth muscle cells: comparison of the DAC-30, DMRIE-C and FuGENE expression systems

Fig. 2 shows the expression of iNOS *in vivo* in the porcine femoral artery: comparison of the DAC-30, FuGENE (Fig. 2A) and DMRIE-C (Fig. 2B) expression systems

Fig. 3 shows the size of the DAC-30/DNA complexes as a function of the lipid/DNA ratio

Fig. 4 shows the expression of iNOS in the porcine femoral artery: effect of the DNA dose on the efficiency of expression *in vivo*

Fig. 5 shows the expression of iNOS *in vitro* in COS-7 cells: efficiency of expression in the transfection solutions L11-L15

Fig. 6 shows the expression of iNOS *in vivo* in the porcine femoral artery: comparison of the efficiency of transfection in solution 11 and 15

Fig. 7 shows the expression of iNOS *in vivo* in the porcine femoral artery: efficiency of transfection in solution 15 after lyophilization and reconstitution

Fig. 8 shows the expression of iNOS *in vivo* in the porcine femoral artery: dependence of the efficiency of transfection on the DNA dose with lyophilized/reconstituted complexes (Fig. 8A: transfection by means of Infiltrator® catheter;

10083248 .072402

Fig. 8B: transfection by means of Infiltrator® catheter and implantation of a Wiktor®-i stent)

10088248 .072402

EXAMPLES

I. Material and methods

1. Transfection systems employed

1.1 DAC-Chol-containing formulations

DAC-30, which is marketed by G.O.T. (Berlin) acts as carrier of the liposomal formulation. DAC-30™ is the proprietary name for a mixture of DAC-cholesterol and the neutral lipid DOPE in the ratio 30:70 (DAC-Chol: DOPE) by weight, which is produced as described in WO 96/20208 and DE 196 23 916. Further mixtures of DAC-Chol and DOPE can be produced in a corresponding way, such as DAC-40 (40:60) or DAC-50 (50:50). The stated concentrations were checked before use.

1.2 Further transfection systems

Also employed, for the purposes of comparison, were DMRIE-C (1,2-dimyristoyloxypropyl-3-dimethylhydroxyethylammonium bromide) from LifeTechnologies (Rockville, Maryland, USA) and FuGENE™ (Boehringer Mannheim).

2. iNOS expression vectors employed

2.1 The plasmid pSCMV-iNOS contains the cDNA sequence of murine iNOS. Its production is described in DE 44 11 402.

2.2 The plasmid pcDNA3-HsiNOS contains the cDNA sequence of human iNOS with a modified 3′ terminus. The cDNA of human iNOS was produced by transcription of isolated RNA from stimulated human hepatocytes into cDNA. The latter were inserted into the cloning vector pGEM-T (Promega) in order firstly to construct the plasmid

pGEM-HsiNOS. The cDNA was cut out of the latter with the restriction endonucleases NotI and ApaI and inserted into the expression vector pcDNA3 (Invitrogen). The plasmid pcDNA3-HsiNOS produced in this way contains the cDNA for human iNOS, with the 3'-terminal DNA sequence coding for the four C-terminal amino acids MSAL having been replaced by a DNA sequence coding for an insert of 20 amino acids (NPAAMAAGSMRRRALFYSVT).

2.3 Plasmid pAH 1 was produced by cutting a fragment 0.9 kb in size containing the 3' terminus of the human iNOS cDNA using the restriction endonuclease SfiI out of the plasmid pcDNA3-HsiNOS. This fragment was replaced by inserting a PCR fragment 0.9 kb in size and containing the 3'-terminal native cDNA sequence of human iNOS into the SfiI cleavage site.

2.4 Plasmid pAH 9 was obtained by cutting a NotI-ApaI fragment which contained the human iNOS cDNA sequence out of pAH 1 and inserting this fragment into the plasmid pAH 7. The latter was produced by modifying pcDNA3 (Invitrogen) by cutting out a BbsI-BsmI fragment comprising 2 kb, filling in the 5' ends and religating, whereby the following deletion was inserted: parts of the BGH polyadenylation sequence, the f1 origin of replication, the SV40 origin of replication, the neomycin resistance gene and parts of the SV40 polyadenylation sequence. In addition, the DNA sequence containing the ampicillin resistance gene was cut out with the restriction enzyme BspHI and replaced by a PCR fragment having the kanamycin resistance gene from the plasmid pZErO-2 (Invitrogen).

3. Solutions which can be employed for stabilizing the lipid/nucleic acid complexes:

Name	Contents
Solution 1 (S1)	300 mM mannitol
Solution 2 (S2)	50 mM mannitol 250 mM sucrose
Solution 3 (S3)	100 mM mannitol 200 mM sucrose
Solution 4 (S4)	150 mM mannitol 150 mM sucrose
Solution 5 (S5)	200 mM mannitol 100 mM sucrose
Solution 6 (S6)	250 mM mannitol 50 mM sucrose
Solution 11 (S11)	0.9% NaCl
Solution 12 (S12)	300 mM glucose
Solution 13 (S13)	300 mM sucrose
Solution 14 (S14)	100 mM sucrose 100 mM NaCl
Solution 15 (S15)	250 mM sucrose 25 mM NaCl
Solution 16 (S16)	300 mM trehalose
Solution 17 (S17)	50 mM trehalose 250 mM sucrose
Solution 18 (S18)	100 mM trehalose 200 mM sucrose
Solution 19 (S19)	150 mM trehalose 150 mM sucrose
Solution 20 (S20)	200 mM trehalose 100 mM sucrose
Solution 21 (S21)	250 mM trehalose 50 mM sucrose
Solution 22 (S22)	50 mM trehalose 250 mM mannitol
Solution 23 (S23)	100 mM trehalose 200 mM mannitol

10088248 .072402

Solution 24 (S24)	150 mM trehalose 150 mM mannitol
Solution 25 (S25)	200 mM trehalose 100 mM mannitol
Solution 26 (S1)	250 mM trehalose 50 mM mannitol

4. Animal models employed

The so-called "Munich minipig" or "Göttingen minipig" were employed for the studies (see Tumbleson, M.E. and Schook, L.B. (1996) supra; Unterberg, C. et al. (1995) supra). The latter experimental animals can be purchased from Göttingen University, Institut für Genetik und Tierzüchtung and from Ellegaard Laboratory Pigs (Dalmose, Denmark). It is possible by using an injection catheter for the transfection to be restricted locally to smooth muscle cells in the media cell layer of blood vessels. The reference quality is the DNA dose per injection. It is also possible where appropriate to choose the plasmid copy number per injection as reference quantity. In the case of plasmid pAH 9 with a size of 6915 Bp, for example, 2 µg correspond to about 2.6×10^{11} copies. The Göttingen minipig was employed in examples 16 and 17, the Munich minipig in the other experiments.

5. Catheter technology employed

The Infiltrator® catheter from IVT (San Diego, CA, USA), model DD140015 (see DE 197 29 769) and the Wiktor®-i stent from Medtronic (Minneapolis, MN, USA), model 6320, were used.

6. Production and transfection of lipoplexes of DAC-30 and plasmid DNA comprising the human iNOS cDNA

6.1 Production of the transfection medium

In the case of solution 15, a solution of 250 mM sucrose and 25 mM NaCl was produced in sterile, pyrogen-free water. This solution was sterilized in a sterile cabinet by filtration through a sterile filter with a pore width of 0.2 µm.

6.2 Preparation of the DAC-30 solution

The vessels containing DAC-30 were stored unopened at -20°C until reconstituted. The reconstitution took place by adding sterile, pyrogen-free water (final concentration 2 mg/ml). The vessels were closed, shaken at room temperature for 30 min and then mixed with a vortex agitator for 2 min. Before each use, the DAC-30 solution was again mixed with a vortex agitator for 30 sec.

6.3 Production of the lipid/nucleic acid complexes from DAC-30 and DNA

All the operations necessary for this were carried out under sterile conditions. The particular amount of DNA was pipetted into half the required final volume of transfection solution. Four times the amount of DAC-30 (based on weight) was added to the other half of the required final volume of transfection solution. The solutions were homogenized using a vortex agitator. The DAC-30 solution was then slowly added dropwise to the DNA solution. The resulting solution was mixed by drawing up into the pipette tip several times or by rotating the closed reaction vessel.

The following production of a DAC-30/DNA mixture with a lipid/DNA ratio of 4:1 (w/w) for administering a therapeutic dose of 2 µg is to serve as a typical example: 7.5 µl of DNA (with a concentration of 1 µg/µl corresponding to 7.5 µg) were adjusted to a final volume of 750 µl by

10088248 .072402

adding transfection solution and homogenized by mixing with the vortex agitator. Four times the amount of DAC-30, i.e. 30 µg (with a concentration of 2 µg/µl corresponding to 15 µl), was likewise taken up in 750 µl of transfection solution and homogenized by mixing with the vortex agitator. Then 750 µl of DAC solution were slowly pipetted dropwise into the 750 µl of DNA solution. The resulting solution contained in a total volume of 1.5 ml lipid/DNA complexes with a DNA concentration of 5 µg/ml. This solution was stable at 4°C for a period of at least 48 h and could either be used immediately or be lyophilized as described below and reconstituted in 1.5 ml of water. It was then possible to inject 400 µl of this solution as described below into the femoral artery of a pig, which corresponds to a therapeutic dose of 2 µg of DNA.

6.4 Lyophilization scheme and procedure

The vessels containing the DAC-30/DNA lipoplexes were frozen under sterile conditions and a freeze drying was carried out in accordance with the following, exemplary scheme. It is evident to the skilled worker that a freeze drying can also be carried out under different conditions:

- 40 hours at a shelf temperature of -37°C and a pressure of 0.140 mbar.
- Temperature of the shelf increased by 10°C/h until 20°C are reached.
- After-drying at 0.01 mbar for 2 h.

6.5 Removal and storage of the samples

The samples were removed under sterile conditions. The lyophilisates were stored at 4°C until used.

6.6 Preparation of the samples for catheter-mediated transfection in vivo

The lyophilisates (containing a defined amount of DAC-30/DNA complexes) were reconstituted in 1.5 ml of sterile, pyrogen-free water immediately before the operation. Redissolution was checked visually.

6.7 Vessel wall transfection by means of Infiltrator catheter *in vivo* (minipig model)

A minipig was intubated, anesthetized and prepared for the surgical operation, which was followed by finding and puncturing the left carotid artery. The guide wire for a port was then positioned, which was followed by installation of the port and administration of 5000 IU of heparin. A survey angiography with static picture was then performed to demonstrate the morphology of the right and left femoral artery. The prospective transfection sites were determined on the basis of this static picture. Next, the guide wire was positioned in the left femoral artery and the correct position of the guide wire was demonstrated by angiography, and the IVT injection catheter was filled and positioned with subsequent inflation of the balloon of the IVT injection catheter to 3.0 bar. An angiography was then performed to check that vessel occlusion was complete. This was followed by manual injection of 300 µl or 400 µl of the reconstituted DAC-30/DNA complexes while monitoring the balloon pressure continuously. The catheter was then removed and optionally a stent implant was positioned exactly at the injection site with 12 bar for 20 s with angiographic monitoring. Implantation of a stent was followed by administration of 1000-2000 IU of heparin. After a final angiography, all the operation wounds were closed.

6.8 Evaluation

The evaluation of the efficiency of transfection took place by immunohistochemical staining of thin sections of the blood vessels removed 3 days after the operation (see DE 197 29 769 with the difference that vessels in which a stent was implanted were embedded in plastic. The thickness of the thin sections was 4 µm for plastic specimens and 10 µm for frozen sections). Alternatively, evaluation of efficacy took place by ultrasound examination of the experimental animals over a period of 3-4 weeks and subsequent histochemical workup of thin sections of the removed vessels.

7. Biochemical detection of iNOS

The formation of nitric oxide by the iNOS was detected by detection of nitrite in the cell culture supernatant as follows: 100 µl of the cell culture supernatant to be investigated were pipetted into a 96-well flat-bottom plate. 50 µl of a 2% strength sulfanilamide solution in 2.5% H_3PO_4 and 50 µl of a 0.2% strength naphthylethylenediamine solution in 2.5% H_3PO_4 were added to each of the samples. After incubation at room temperature for 5 min, the absorption of the samples at 540 nm was compared with a standard series ($NaNO_2$ in a concentration of 0 µM, 1 µM, 2.5 µM, 5.0 µM, 10 µM, 20 µM and 40 µM in cell culture medium).

II. Examples

1. Comparison of liposomal transfection compositions for expression of nitric oxide synthase *in vitro*: DAC-30, DMRIE-C and FuGENE™

Experiment: Transfection solutions consisting of cationic liposomes and the plasmid pSCMV-iNOS (murine iNOS cDNA) were produced. The transfection solutions were produced by mixing a constant dose of 8 µg of DNA with the cationic liposomes DAC-30 (1:5, w/w), DMRIE-C (1:2, w/v) and FuGENE™ (1:1, w/v) in BSS buffer (137 mM NaCl, 5.4 mM KCl, 10 mM Tris/Cl pH 7.6). Smooth muscle cells from the porcine aorta were cultivated in cell culture dishes each with 6 wells. 500 µl of transfection solution (containing DNA in a concentration of 16 µg/ml) were added to the cells in each well and incubated at 37°C for 1 h and, after replacement of the solutions by cell culture medium, the cells were cultivated for a further 48 h. The amount of accumulated nitrite from the cell culture supernatant of the transfected cells was then determined by comparison with a control transfection without DNA (see I.7).

Result: The expression of inducible nitric oxide synthase (iNOS) *in vitro* depended on the lipid used. A transfection solution containing DAC-30/iNOS DNA led to lower expression *in vitro* of inducible nitric oxide synthase than did transfection solutions containing FuGENE/iNOS DNA or DMRIE-C/iNOS DNA (see Fig. 1).

2. Comparison of liposomal transfection compositions for expression of nitric oxide synthase *in vivo*: DAC-30, DMRIE-C and FuGENE™

Experiment: Transfection solutions consisting of cationic liposomes and the plasmid pSCMV-iNOS (murine

iNOS cDNA) were produced. The transfection solution was produced by mixing a dose of 10 μg of plasmid DNA with DAC-30 in the ratio 1:5 (w/w), with DMRIE-C in the ratio 1:2 (w/v), with FuGENE™ in the ratio 1:1 (w/v) in BSS buffer (137 mM NaCl, 5.4 mM KCl, 10 mM Tris/Cl pH 7.6). In each case two sites in the right and left femoral artery of the pig were transfected *in vivo* using an Infiltrator® catheter (IVT) with the solutions produced as described. After an experimental period of 3 days, the transfected vessel sections were removed. Evaluation of the immunohistochemical staining of thin sections of the removed vessels with a monoclonal antibody against inducible nitric oxide synthase took place qualitatively, comparing with an untreated control vessel.

Result: Transfection solutions containing DAC-30/iNOS DNA showed a distinctly better efficiency of transfection *in vivo* than did liposome/DNA complexes containing FuGENE™/iNOS DNA or DMRIE-C/iNOS DNA. This was a very surprising result in view of the *in vitro* data obtained in example 1 (see Fig. 2.A and 2.B).

3. DAC-30: Expression and toxicity *in vitro* as a function of the lipid/DNA ratio

Experiment: Smooth muscle cells from the porcine aorta (see example 1) were cultivated in cell culture dishes each with 6 wells and transfected with liposome/DNA complexes consisting of DAC-30 and the plasmid pAH 1 (human iNOS cDNA). To produce the transfection solution (0.9% NaCl, 2 mM $CaCl_2$), DAC-30 was mixed with a constant dose of 4 μg of DNA (corresponding to a concentration of 8 μg/ml) in the ratio 2:1, 4:1, 5:1, 6:1 and 8:1. As an alternative to this, a DNA dose of 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml was employed to produce the transfection solution with a constant liposome/DNA ratio of 5:1. 500 μl of transfection

solution were added to the cells in each well and incubated at 37°C for 1 h and, after replacement of the solutions by cell culture medium, cultivated for a further 48 h. After 48 h, the amount of accumulated nitrite in the cell culture supernatant of the transfected cells was determined, comparing with a control transfection without DNA in the transfection solution (0.9% NaCl, 2 mM $CaCl_2$) and with untransfected cells (medium) (see I.7). The cytotoxicity of the transfection solution was tested in parallel by a vitality test (uptake of neutral red solution).

Result: Expression of inducible nitric oxide synthase by liposomal complexes containing DAC-30/iNOS DNA depended on the lipid/DNA ratio in the production of the complexes. With a constant dose of 4 µg of iNOS plasmid (concentration 8 µg/ml), expression of the transgene was optimal for a lipid/DNA ratio of 5:1. At the same time, these liposomal complexes showed a low cytotoxicity on transfection *in vitro*. With a suitable liposome/DNA ratio of 5:1, liposome/DNA complexes containing DAC-30/iNOS DNA showed an optimal expression efficiency at a DNA concentration of 4 µg/ml to 8 µg/ml and a low cytotoxicity up to a DNA concentration of 8 µg/ml. (Data not shown).

4. Size of complexes as a function of the lipid/DNA ratio

Experiment: A transfection solution consisting of complexes of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in saline solution (0.9% NaCl/2 mM $CaCl_2$) was produced. The liposome/DNA complexes were produced by employing DAC-30 in the ratio by weight 2:1, 3:1, 4:1, 5:1, 6:1 and 8:1 to the plasmid DNA with a constant DNA concentration of 6.7 µg/ml DNA. The size of the complexes was determined by photon correlation spectroscopy (Zetasizer).

Result: Lipid/DNA mixtures containing DAC-30/iNOS DNA with a constant DNA concentration of 6.7 µg/ml formed complexes of increasing particle size depending on the lipid/DNA ratio (2:1, 3:1, 4:1, 5:1, 6:1, 8:1) (see Fig. 3).

5. Size of complexes as a function of amount of DNA influences the expression efficiency *in vivo*

Experiment: Lipid/DNA mixtures consisting of complexes of DAC-30 and the plasmid pcDNA3-HsiNOS (human iNOS cDNA with modified 3' terminus) in a saline solution (0.9% NaCl, 2 mM $CaCl_2$) were produced. Liposome/DNA complexes were produced by employing DAC-30 in the ratio 5:1 to the plasmid DNA with a DNA dose of 2 µg (concentration 6.7 µg/ml) or 10 µg of DNA (concentration 33.3 µg/ml). Immediately after production, the size of the formed complexes was determined by photon correlation spectroscopy (Zetasizer). Transfection solutions produced in this way were used to transfect in each case two sites in the right and left femoral artery of a pig *in vivo* using an Infiltrator™ catheter. After an experimental period of 3 days, the transfected vessel sections were removed. Evaluation of the immunohistochemical staining of thin sections of the removed vessels with a monoclonal antibody against inducible nitric oxide synthase took place qualitatively, comparing with an untreated control vessel.

Result: The freshly produced lipid/DNA mixtures containing DAC-30/iNOS DNA in the ratio 5:1 formed complexes with a smaller particle size (about 580 nm) with a dose of 2 µg of iNOS plasmid (corresponding to a concentration of 6.7 µg/ml) than with a dose of 10 µg of iNOS plasmid (corresponding to a concentration of 33.3 µg/ml (about 1000 nm). On transfection into the vessel wall of the porcine femoral artery *in vivo*,

these mixtures showed a better efficiency of transfection with a dose of 2 µg of iNOS plasmid than with a dose of 10 µg of iNOS plasmid. The better efficiency of transfection with a smaller dose *in vivo* (see Fig. 4) was surprising and correlates with a smaller particle size of the DNA/liposome complexes with this dose.

6. Efficacy: prevention of neointima formation after iNOS DNA/DAC-30 transfection

<u>Experiment</u>: Transfection solutions consisting of DAC-30 and 0.7 µg (corresponding to a DNA concentration of 2.3 µg/ml) of the plasmid pAH1 (human iNOS cDNA) or of the control plasmid pcDNA3 (without cDNA insert) in the ratio 5:1 in a saline solution (0.9% NaCl/2 mM $CaCl_2$) were produced. In each case two sites in the right and left femoral artery of a pig were transfected *in vivo* using an Infiltrator® catheter (IVT) with the solutions produced as described. A Wiktor®-i stent (Medtronic) was subsequently placed at each transfection site to induce neointima formation. After an experimental period of 6 weeks, the transfected vessel sections were removed. Evaluation of thin sections of the removed vessels took place quantitatively by morphometry, comparing with the control (plasmid pcDNA3) (n = 3).

<u>Result</u>: Expression of iNOS after transfection of solutions containing DAC-30/iNOS DNA into the porcine femoral artery *in vivo* brought about a reduction in neointima formation (determined as ratio of neointima to media) of about 20% (data not shown).

7. Time-dependent stability of lipid/DNA complexes

<u>Experiment</u>: Lipid/DNA mixtures consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in the ratio 4:1 and 5:1 with an increasing DNA concentration of

10088248.072402

2.3 µg/ml, 6.7 µg/ml and 13.3 µg/ml in solution 11 (0.9% NaCl) were produced. The size of the resulting liposome/DNA complexes was determined immediately after production and after standing at 4°C for 3 h by photon correlation spectroscopy (Zetasizer).

<u>Result</u>: Lipid/DNA mixtures containing DAC-30/iNOS DNA with a lipid/DNA ratio of 4:1 formed complexes in a defined size range from about 540 to 700 nm, which also remained constant over a period of 3 h at 4°C with an increasing DNA concentration of 2.3 µg/ml, 6.7 µg/ml and 13.3 µg/ml. This constant particle size is a measure of the stability of the complexes. By contrast, with a lipid/DNA ratio of 5:1, the size of the complexes with an increasing amount of DNA of 2.3 µg/ml, 6.7 µg/ml and 13.3 µg/ml increased over a period of 3 h at room temperature as follows: with 2.3 µg/ml on average by a factor of 2 from about 650 nm to about 1280 nm, with 6.7 µg/ml by a factor of 3 from about 740 nm to about 2200 nm and with 13.3 µg/ml by a factor of 2.5 from about 940 nm to about 2300 nm. This time-dependent increase in size means that the complexes were unstable. (Data not shown).

8. Time-dependent stability of lipid/DNA complexes in various transfection solutions

<u>Experiment</u>: A lipid/DNA mixture consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in the ratio 4:1 with a constant DNA concentration of 5 µg/ml was produced. The liposome/DNA complexes were produced in various solutions: L 12 (300 mM glucose), L 13 (300 mM sucrose), L 14 (100 mM sucrose, 100 mM NaCl), L 15 (250 mM sucrose, 25 mM NaCl) or L 11 (0.9% NaCl). The size of the liposome/DNA complexes was determined either immediately after production or after storage at 4°C for 3 h, 24 h and 48 h by photon correlation spectroscopy (Zetasizer).

10088248 .072402

Result: Lipid/DNA mixtures containing DAC-30/iNOS DNA with a lipid/DNA ratio of 4:1 and a DNA concentration of 5 μg/ml formed complexes in a defined size range from about 460 to 610 nm. This size of the complexes remained substantially constant in the transfection solutions L 13, L 14 and L 15 over a period of 3 h to 48 h at 4°C (increased by a factor of about 1.2 in solution 13, by a factor of about 1.4 in solution 14 and by about 1.1 in solution 15), i.e. the formed complexes were stable. By contrast, DAC-30/DNA complexes produced in L 11 showed a marked increase in the particle size, by about a factor of 2.5, over a period of 3 h to 48 h, i.e. the formed complexes were unstable. A slight increase in the particle size, by a factor of about 1.6, was also found in solution 12. (Data not shown).

9. Efficiency of expression *in vitro* in various transfection solutions

Experiment: A transfection solution consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in solutions L 12 (300 mM glucose), L 13 (300 mM sucrose), L 14 (100 mM sucrose, 100 mM NaCl), L 15 (250 mM sucrose, 25 mM NaCl) or L 11 (0.9% NaCl) was produced. To produce the transfection solution, DAC-30 was added in the ratio 4:1 to a constant amount of 2 μg of DNA (corresponding to a concentration of 5 μg/ml). The liposome/DNA complexes were transfected into COS-7 cells immediately after production. After 48 h, the nitrite formed was detected in the cell culture supernatant (see I.7). Means of 3 independent experiments are shown, and the level of the expression was standardized using the measurements in a neutral red test (see Fig. 5).

Result: Expression of inducible nitric oxide synthase by lipid/DNA mixtures containing DAC-30/iNOS DNA was

10088248.072402

substantially independent of the composition of the transfection solution. Solutions L 12, L 13, L 14 and L 15 showed an efficiency of expression comparable with that of L 11 at a constant lipid/DNA ratio of 4:1 and a dose of 2 μg of DNA (see Fig. 5).

10. Efficiency of transfection *in vivo* in solution 15 compared with solution 11

<u>Experiment</u>: Transfection solutions consisting of DAC-30 and 2 μg of the plasmid pAH 1 (human iNOS cDNA) in the ratio 5:1 were produced in solution 15 (250 mM sucrose, 25 mM NaCl) and solution 11 (0.9% NaCl). In each case two sites in the right and left femoral artery of a pig were transfected *in vivo* using an Infiltrator™ catheter (IVT) with the solutions produced as described. After an experimental period of 3 days, the transfected vessel sections were removed. Quantitative evaluation of the immunohistochemical data: plotting of the means from 5 sections located in the middle of the transfection site (distance 500 μm in each case)(n = 2 pigs).

<u>Result</u>: Liposome/DNA complexes containing DAC-30/iNOS DNA in L 15 showed a better efficiency of transfection *in vivo* with a dose of 2 μg of iNOS plasmid than did complexes produced in L 11. The better efficacy of transfection solution L 15 *in vivo* was not to be expected from the available data generated *in vitro* (see example 10) (see Fig. 6).

11. Short-term stability of DAC-30/DNA complexes in solution 15

<u>Experiment</u>: A lipid/DNA mixture consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in the ratio 4:1 was produced in solution 15 (250 mM sucrose, 25 mM NaCl). The DNA concentration was 2.5 μg/ml, 10 μg/ml,

25 µg/ml or 50 µg/ml. The particle size of the lipid/DNA mixtures was determined immediately after production or reconstitution of the lyophilized complexes (0 h), and after standing at 4°C for 3 h, by photon correlation spectroscopy (Zetasizer).

Result: Lipid/DNA mixtures containing DAC-30/iNOS DNA with a lipid/DNA ratio of 4:1 and an increasing DNA concentration of 2.5 µg/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml in solution 15 showed a constant particle size over a period of 3 h (increase in size less than 15%). This constant particle size over a period of at least 3 h is advantageously maintained stably even after lyophilization of the complexes and subsequent reconstitution in sterile water. The lyophilization thus has no effect on the short-term stability of the liposome/DNA complexes. (Data not shown).

12. Long-term stability of DAC-30/DNA complexes in solution 15

Experiment: A lipid/DNA mixture consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in the ratio 4:1 was produced in solution 15 (250 mM sucrose, 25 mM NaCl). The DNA concentration was 2.5 µg/ml, 5 µg/ml or 10 µg/ml. The particle size of the produced complexes was determined by photon correlation spectroscopy (Zetasizer) immediately (0 h) and after standing for 3 h, 24 h and 48 h. The transfection solutions produced in this way contained a total dose of 1 µg, 2 µg or 4 µg of plasmid DNA and were transfected into COS-7 cells immediately (0 h) or after standing for 3 h, 24 h and 48 h. The accumulated nitrite in the cell culture supernatant was detected 48 h after transfection (see I.7). The amount of cellular protein was determined in parallel with this (n=3).

<u>Result</u>: Lipid/DNA mixtures containing DAC-30/iNOS DNA with a constant lipid/DNA ratio of 4:1 and an increasing DNA concentration of 2.5 μg/ml, 5 μg/ml and 10 μg/ml in solution 15 showed a constant average particle size of about 480 nm, about 500 nm and about 510 nm over a period of 48 h. Transfection of COS-7 cells with these lipid/DNA mixtures brought about a constant expression of the transgene after the transfection solution had been standing for up to 48 h. DAC-30/DNA complexes produced in solution 15 advantageously showed a constant particle size and expression efficiency, which was maintained even with increasing DNA concentration, after the transfection solution had been standing for up to 48 h. (Data not shown).

13. Efficiency of transfection *in vivo*: effect of the DNA dose

<u>Experiment</u>: Transfection solutions consisting of DAC-30 and 0.5 μg, 1 μg or 2 μg of the plasmid pAH 9 (human iNOS cDNA) or of the control plasmid pAH 7 (without cDNA insert) in the ratio 4:1 were produced in solution 15 (250 mM sucrose, 25 mM NaCl). In each case two sites in the right and left femoral artery of a pig were transfected *in vivo* using an Infiltrator™ catheter (IVT) with the solutions produced as described. A Wiktor®-i stent (Medtronic) was then placed at each transfection site. After an experimental period of 3 days, the transfected vessel sections were removed. Evaluation of the immunohistochemical staining of thin sections of the removed vessels with a monoclonal antibody against inducible nitric oxide synthase took place quantitatively by morphometry, comparing with the control (plasmid pAH 7 or untreated vessel).

<u>Result</u>: Liposome/DNA complexes containing DAC-30/iNOS DNA in solution 15 showed a better efficiency of

10088248.072402

transfection *in vivo* with a dose of 2 µg of iNOS plasmid than with 0.5 µg and 1 µg (about 43% compared with respectively about 31.3% and 13%, based on the transfected media as a proportion of the total media). (Data not shown).

14. Efficiency of transfection *in vivo* in solution 15 after lyophilization and reconstitution

<u>Experiment</u>: A transfection solution consisting of DAC-30 and the plasmid pAH 9 (human iNOS cDNA) in the ratio 4:1 was produced in solution 15. In each case one site in the right or left femoral artery of a pig was transfected *in vivo* by means of an Infiltrator® catheter employing freshly prepared transfection solution (dose 2 µg of DNA) or transfection solution which had been lyophilized and reconstituted in sterile water (0.5; 1; and 2 µg of DNA). A Wiktor®-i stent (Medtronic) was then placed at each transfection site. After an experimental period of 3 days, the transfected vessel sections were removed. Evaluation of the immunohistochemical staining of thin sections of the removed vessels with a monoclonal antibody against inducible nitric oxide synthase took place quantitatively by morphometry.

<u>Result</u>: A transfection solution containing DAC-30/iNOS DNA in solution 15 (dose of 2 µg of DNA) surprisingly showed an efficiency of transfection *in vivo* as reconstituted lyophilisate which was better by a factor of 1.5 than freshly prepared complexes (Fig. 7; from left to right: freshly produced transfection solution (2 µg); control (2 µg); reconstituted transfection solution (0.5; 1; and 2 µg of DNA)).

15. Efficiency of transfection *in vivo* with lyophilized/reconstituted complexes: DNA dose-dependence

Experiment: Transfection solutions consisting of DAC-30 and 0.7 μg, 1 μg, 1.5 μg, 2 μg or 4 μg of the plasmid pAH 9 (human iNOS cDNA) or the control plasmid pAH 7 (without cDNA insert) in the ratio 4:1 were produced. The DNA/liposome complexes were produced in solution 15, lyophilized and reconstituted in sterile water immediately before transfection. In each case two sites in the right and left femoral artery of a pig were transfected *in vivo* with the solutions produced as described using an Infiltrator™ catheter (Fig. 8A). The experiment was carried out alternatively with implantation of a Wiktor®-i stent (Fig. 8B). After an experimental period of 3 days, the vessels were removed. Quantitative evaluation of the immunohistochemical data of the experiments with and without stent implantation: proportion of transfected media as a percentage from the means of 10 μm sections at distances each of 1 mm over the entire transfected vessel section.

Result: Liposome/DNA complexes containing iNOS DNA/ DAC-30 showed a better efficiency of transfection *in vivo* as reconstituted lyophilisate in a dose of 2 μg of plasmid DNA than in a dose of 0.7 μg, 1 μg, 1.5 μg or 4 μg (Fig. 8A). The better efficiency of transfection with a dose of 2 μg *in vivo* was unaffected by implantation of a stent (see Fig. 8B; implantation of a Wiktor®-i stent).

16. Efficacy *in vivo*: prevention of neointima formation by transfection of 1 μg of iNOS

Experiment: A transfection solution consisting of DAC-30 and 1 μg of the plasmid pAH 9 (human iNOS cDNA) or of the control plasmid pAH 7 (without cDNA insert) in the ratio 4:1 was produced in solution 15. The solution was lyophilized and reconstituted in sterile water immediately before transfection into in each case

one site in the right and left femoral artery of a pig *in vivo* using an Infiltrator® catheter. A Wiktor®-i stent (Medtronic) was then placed at each transfection site to induce neointima formation. After an experimental period of 28 days, the transfected vessel sections were removed. Evaluation was carried out by intravascular ultrasonic examination of the transfected vessel sections and quantified the reduction in the neointima formed after treatment with the iNOS plasmid compared with the control plasmid as ratio of the plaque area to the total vessel area (n=8).

Result: Liposome/DNA complexes produced in solution 15 and containing a dose of 1 µg of iNOS DNA/DAC-30 brought about a reduction in neointima formation in the pig restenosis model *in vivo* of about 42% after lyophilization and reconstitution in sterile water. (Data not shown).

17. Efficacy *in vivo*: prevention of neointima formation by transfection of 0.25 µg, 0.5 µg or 1 µg of iNOS

A transfection solution consisting of DAC-30 and 0.25 µg, 0.5 µg or 1 µg of the plasmid pAH 9 (human iNOS cDNA) or of the control plasmid pAH 7 (without cDNA insert) in the ratio 4:1 was produced in solution 15. The solution was lyophilized and reconstituted in sterile water immediately before transfection into in each case one site in the right and left femoral artery of a pig *in vivo* using an Infiltrator® catheter. A Wiktor®-i stent (Medtronic) was then placed at each transfection site to induce neointima formation. After an experimental period of 28 days, the transfected vessel sections were removed. Evaluation was carried out by intravascular ultrasonic examination of the transfected vessel sections and quantified the reduction in the neointima formed after treatment with

10088248 .072402

the iNOS plasmid compared with the control plasmid as ratio of the plaque area to the total vessel area.

18. Prevention of transplant arteriosclerosis *in vivo* by gene therapy with HO-1

Rabbits weighing 3-3.5 kg were used for the experiment on prevention of transplant arteriosclerosis. The animals are anesthetized for the surgical operation, which is carried out under sterile conditions. The gene is transferred into the donor vessel using an Infiltrator® catheter which is introduced via the exposed right carotid artery through a port by means of a guide wire into the iliac artery. Injection of the HO-1 expression plasmid into the vessel wall of the iliac artery by means of an Infiltrator® catheter takes place with a low balloon pressure (about 0.6 atm). This involves 100-150 μl of transfection solution consisting of DAC-30 and an HO-1 expression plasmid in the ratio 4:1 in solution 15 being injected into the vessel wall within about 30 seconds. The location of the inflated catheter is checked by angiography. After removal of the catheter, the transfected vessel segment is removed and inserted at the corresponding site into the iliac artery of an allogenic recipient rabbit. After 2-4 months, a histochemical investigation is carried out to find whether the development of arteriosclerotic lesions in the implanted vessels (intimal hyperplasia, leukocyte infiltration) can be reduced by comparison with control-treated animals.

19. Induction of growth of collateral vessels *in vivo* by gene therapy with MCP-1

Rabbits (New Zealand White rabbits) weighing 3-3.5 kg are used for the experiment on induction of growth of collateral vessels. The animals are anesthetized for the surgical operation, which is carried out under

sterile conditions. The exposed femoral artery is tied off by two ligatures 1.5-2 cm apart in such a way that the branches of the deep femoral artery, the lateral circumflex femoral artery and the circumflex abdominal artery remain patent. An Infiltrator® catheter which is introduced via the exposed right carotid artery through a port by means of a guide wire into the femoral artery is used for the gene transfer. The MCP-1 plasmid is injected into the vessel section located proximal of the ligature using an Infiltrator® catheter with a low balloon pressure (about 0.6 atm). This involves 100-150 µl of transfection solution consisting of DAC-30 and an MCP-1 expression plasmid in the ratio 4:1 in solution 15 being injected into the vessel wall within about 30 seconds. The location of the inflated catheter is checked by angiography. After removal of the catheter, all the surgical wounds are closed. After 7 days, an angiographic investigation is carried out into whether more collaterals have formed than in control-treated animals. Histological methods are used to investigate the proliferation of endothelial cells and smooth muscle cells in the vascular system of the treated tissue compared with control-treated animals.

20. Induction of growth of collateral vessels *in vivo* by gene therapy with MCP-1/GM-CSF

Rabbits (New Zealand White rabbits) weighing 3-3.5 kg are used for the experiment on induction of growth of collateral vessels. The animals are anesthetized for the surgical operation, which is carried out under sterile conditions. The exposed femoral artery is tied off by two ligatures 1.5-2 cm apart in such a way that the branches of the deep femoral artery, the lateral circumflex femoral artery and the circumflex abdominal artery remain patent. About 7-21 days after the vessel occlusion, a gene transfer is carried out with plasmid DNA which leads to expression of MCP-1 or GM-CSF in the

vessel wall. An Infiltrator® catheter which is introduced via the exposed right carotid artery through a port by means of a guide wire into the femoral artery is used for the gene transfer. The plasmid DNA is injected into the vessel section located proximal of the ligature using an Infiltrator® catheter with a low balloon pressure (about 0.6 atm). This involves 100-150 μl of transfection solution consisting of DAC-30 and plasmid DNA in the ratio 4:1 in solution 15 being injected into the vessel wall within about 30 seconds. The location of the inflated catheter is checked by angiography. After removal of the catheter, all the surgical wounds are closed. After a further 7 days, an angiographic investigation is carried out into whether more collaterals have formed than in control-treated animals. Histological methods are used to investigate the proliferation of endothelial cells and smooth muscle cells in the vascular system of the treated tissue compared with control-treated animals.

10088248 .072402

Patent claims

1. A pharmaceutical composition in the form of a nucleic acid/lipid complex, comprising

(a) at least one cationic lipid (CL);
(b) at least one non-cationic lipid (NCL);
(c) at least one nucleic acid (N) coding for a protein for the treatment of vascular disorders, in particular a protein with vasodilating and/or angiogenic properties; and
(d) where appropriate, suitable excipients and/or additives;

where the cationic lipid (CL) comprises a group which is derived from cholesterol and to which at least one cationic amino group selected from primary, secondary, tertiary amino group and/or a quaternary ammonium salt is linked via a connecting group selected from carboxamides and carbamoyls, and a spacer consisting of a linear or branched alkyl group having 1 to 20 carbon atoms, and where the size of the nucleic acid/lipid complexes is in a range of about 300-800 nm.

2. A pharmaceutical composition as claimed in claim 1, where the size of the nucleic acid/lipid complexes is in a range of about 350-550 nm.

3. A pharmaceutical composition as claimed in claim 1 or 2, where the nucleic acid (N) codes for one of the isoforms of nitric oxide synthase (NOS), of hemoxygenase (HO), of monocyte chemoattractant protein (MCP), or a variant of one of these proteins.

4. A pharmaceutical composition as claimed in any of claims 1 - 3, where the nucleic acid codes for inducible nitric oxide synthase (iNOS), hemoxygenase-1 (HO-1), monocyte chemoattractant protein-1 (MCP-1), or

a variant thereof, preferably for the human form in each case.

5. A pharmaceutical composition as claimed in any of claims 1 - 4, where the cationic lipid (CL) is 3β-[N-(N,N'-dimethylaminoethane)carbamoyl]cholesterol (DAC-Chol) or 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

6. A pharmaceutical composition as claimed in any of claims 1 - 5, where the non-cationic lipid (NCL) is a lipid selected from at least one phosphatidylcholine, at least one phosphatidylethanolamine and/or cholesterol.

7. A pharmaceutical composition as claimed in claim 6, where the phosphatidylethanolamine is a diacylphosphatidylethanolamine with a chain length of 10-28 carbon atoms, preferably dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE) and/or dioleylphosphatidylethanolamine (DOPE).

8. A pharmaceutical composition as claimed in claim 7, where the cationic lipid (CL) is DAC-Chol and the non-cationic lipid (NCL) is DOPE, preferably in a ratio of DAC-Chol to DOPE by weight of from about 10:90 to about 90:10, particularly preferably of about 30:70.

9. A pharmaceutical composition as claimed in any of claims 1 - 8, where the composition has been obtained by using total lipid composed of (CL) and (NCL) to nucleic acid (N) in the ratio of from about 1:1 to about 10:1, preferably about 4:1 or about 5:1, in each case based on weight.

10088249 .072402

10. A pharmaceutical composition as claimed in any of claims 1 - 9, where the composition is in the form of a solution or lyophilisate.

11. A pharmaceutical composition as claimed in any of claims 1 - 10, where the excipient mentioned is a stabilizing agent, in particular at least one sugar, at least one polyhydric alcohol and/or at least one inorganic salt.

12. A pharmaceutical composition as claimed in any of claims 1 - 11, where the additive mentioned is at least one molecule specifically recognizing the target cells and/or at least one molecule facilitating gene transfer into the cells.

13. A method for the production of the pharmaceutical composition as claimed in any of claims 1 - 12, characterized by the following steps:

(i) provision of a mixture of a cationic lipid (CL) as set forth in any of claims 1 - 12 and a non-cationic lipid (NCL) as set forth in any of claims 1 - 12, and provision of a nucleic acid (N) as set forth in any of claims 1 - 12;
(ii) mixing of the mixture of (CL) and (NCL) with the nucleic acid (N);
(iii) where appropriate lyophilization; and
(iv) where appropriate reconstitution.

14. A method as claimed in claim 13, where in step (ii) the total lipid composed of (CL) and (NCL) and the nucleic acid (N) are mixed in the ratio of from about 1:1 to about 10:1, preferably about 4:1 or about 5:1, in each case based on weight.

15. A method as claimed in claim 13 or 14, where in step (i) the provision of the mixture of (CL) and (NCL)

and/or the provision of the nucleic acid (N) takes place with use of a stabilizing agent, in particular at least one sugar, at least one polyhydric alcohol and/or at least one inorganic salt.

16. A method as claimed in claim 15, where the stabilizing agent is used in the form of an isoosmotic aqueous solution.

17. A pharmaceutical composition obtainable by the method as claimed in any of claims 13 - 16.

18. The use of the pharmaceutical composition as claimed in any of claims 1 - 12 or 17 for producing a pharmaceutical for use in gene therapy including a combination therapy with pharmacological active substances.

19. The use as claimed in claim 18 for the treatment of vascular disorders, genetically related disorders and/or disorders which can be treated by gene transfer, including prevention thereof.

20. The use as claimed in claim 18 or 19 for the treatment and prevention of peripheral and/or coronary vascular disorders.

21. The use as claimed in claim 20, where the vascular disorder is stenosis of vessels including vessel transplants, restenosis after percutaneous transluminal angioplasty (PTA) of coronary and/or peripheral vessels, a disorder resulting from hypoperfusion of tissues, coronary heart disease, myocardial infarction, vascular arteriosclerosis and/or a disorder which leads to rejection of vessel and/or organ transplants.

22. The use as claimed in any of claims 18 - 21 for local somatic gene therapy.

23. The use as claimed in claim 22, where the gene therapy takes place with use of a catheter, in particular an Infiltrator catheter.

24. The use as claimed in claim 22 or 23, where the pharmaceutical composition is administered in a total dose in a range of about 0.1 - 20 μg, preferably about 0.5 - 10 μg, particularly preferably about 1 - 5 μg, in each case based on the total amount of nucleic acid per administration.

25. The use of an isoosmotic composition comprising at least one mono- and/or disaccharide and/or at least one polyhydric alcohol and/or at least one inorganic salt for stabilizing nucleic acid/lipid complexes in solution.

26. The use of an isoosmotic composition comprising at least one mono- and/or disaccharide and/or at least one polyhydric alcohol and/or at least one inorganic salt for stabilizing nucleic acid/lipid complexes during lyophilization and/or reconstitution.

27. The use as claimed in claim 25 or 26, where the disaccharide is sucrose and the inorganic salt is sodium chloride.

Abstract

The invention relates to a pharmaceutical composition in the form of a nucleic acid/lipid complex, comprising at least one cationic lipid, at least one non-cationic lipid, at least one nucleic acid coding for a protein for the treatment of vascular disorders, in particular a protein with vasodilating and/or angiogenic properties, and where appropriate, suitable excipients and/or additives, where the cationic lipid (CL) comprises a group which is derived from cholesterol and to which at least one cationic amino group selected from primary, secondary, tertiary amino group and/or a quaternary ammonium salt is linked via a connecting group selected from carboxamides and carbamoyls, and via a spacer consisting of a linear or branched alkyl group having 1 to 20 carbon atoms, and where the size of the nucleic acid/lipid complexes is in a range of about 300-800 nm. The invention also relates to the production of the pharmaceutical composition and to its use in gene therapy.

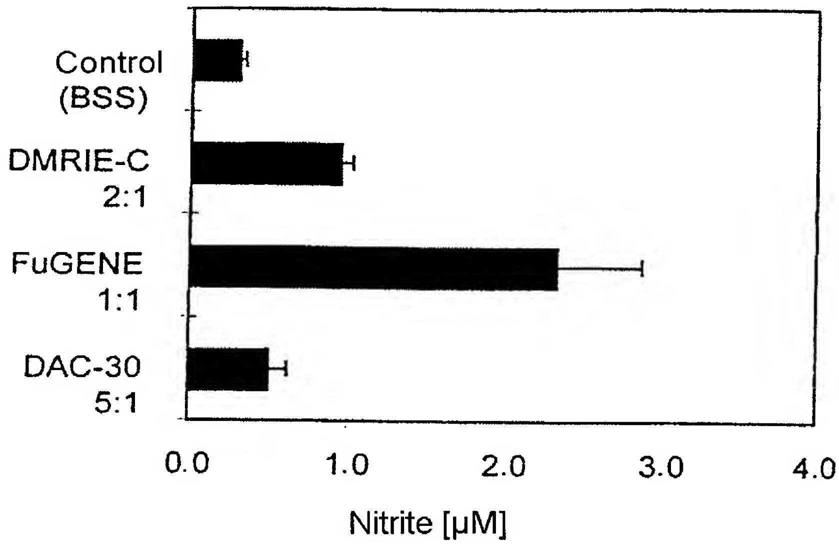


Fig.1

[redacted]

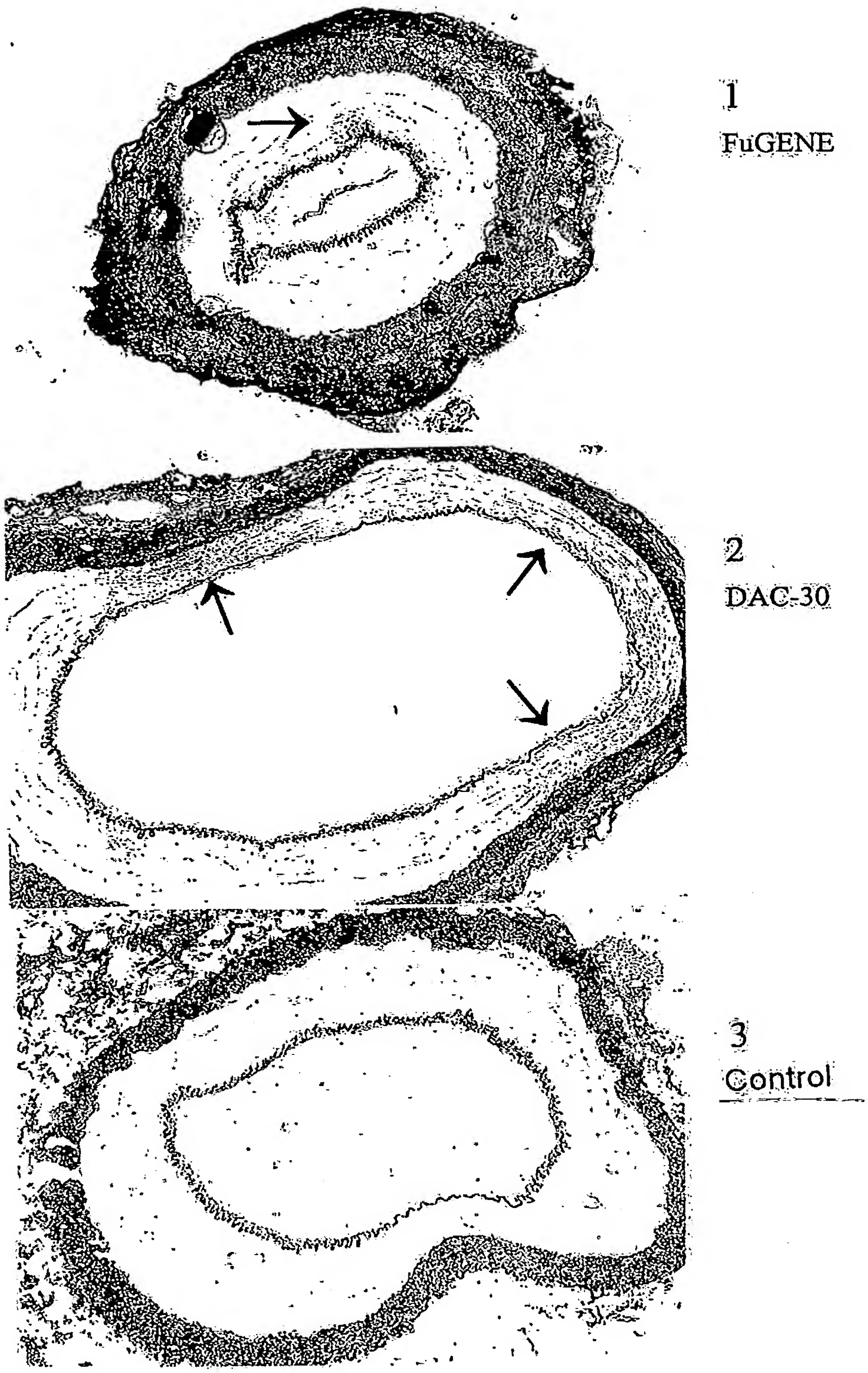


Fig. 2A

1
DMRIE-C

2
DAC-30

3
Control

Fig. 2B

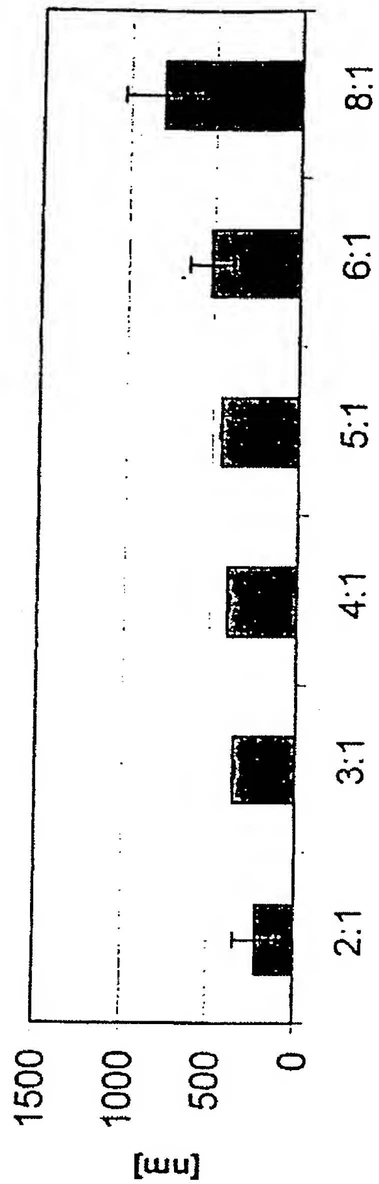


Fig. 3

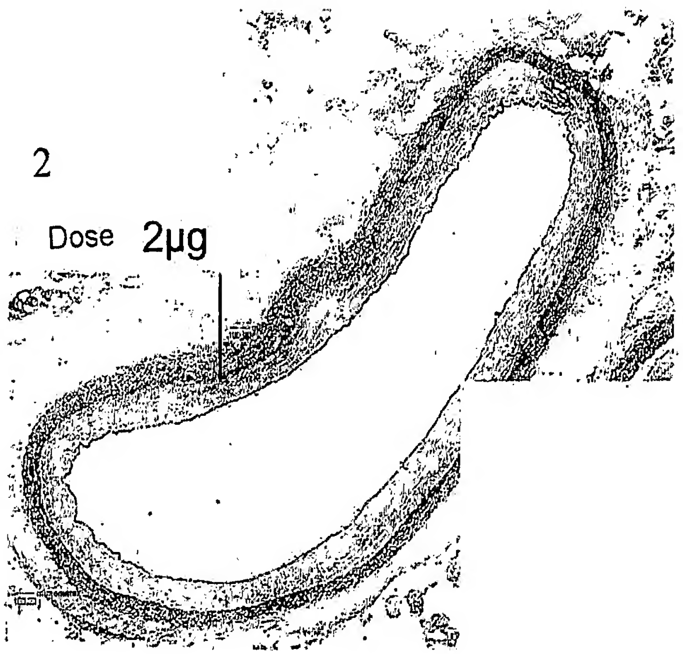
Fig. 4

1

Control

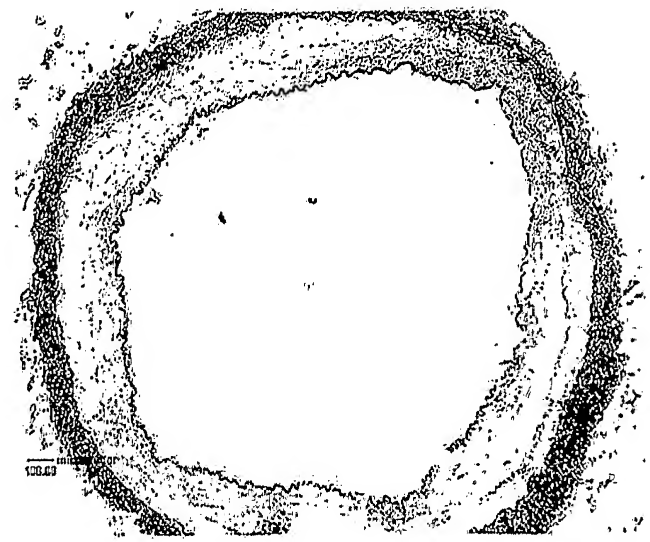
2

Dose 2µg



3

Dose 10 µg



10/088248

Fig. 5

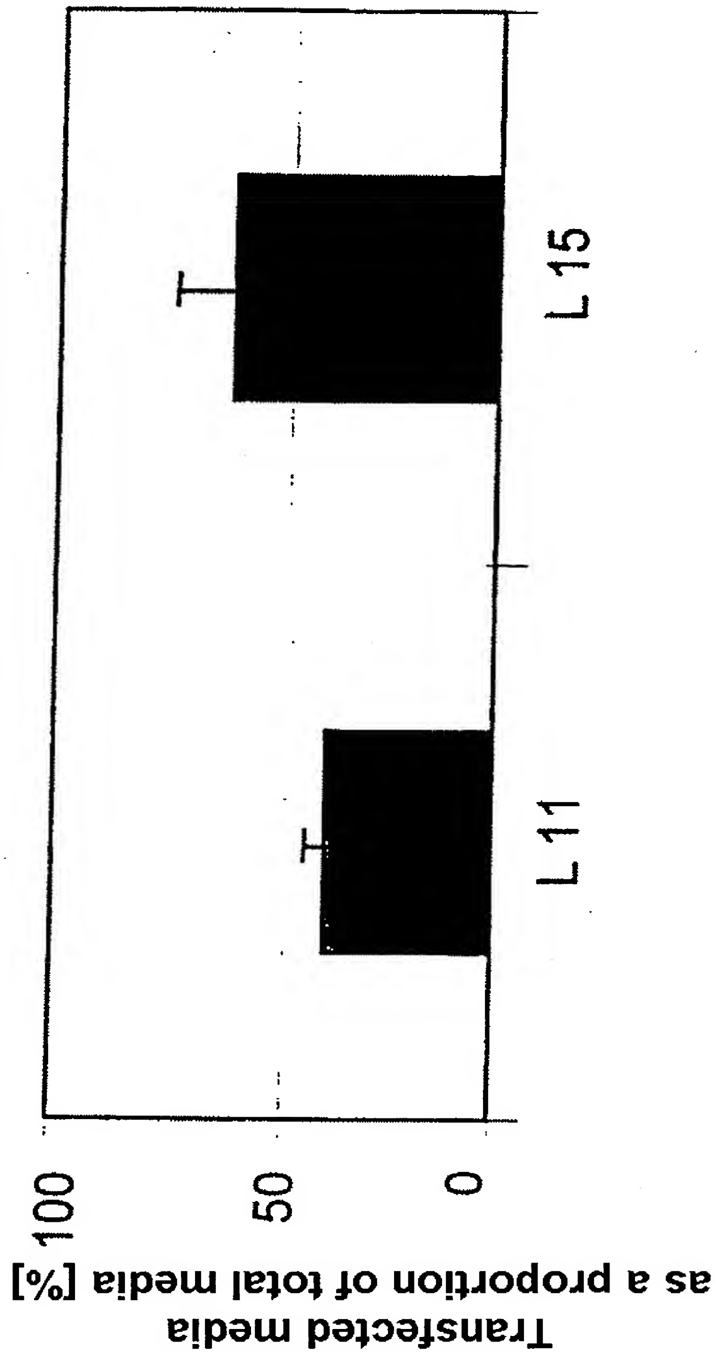

Transfected media
as a proportion of total media [%]
100
50
0
L 11
L 15

Fig. 6

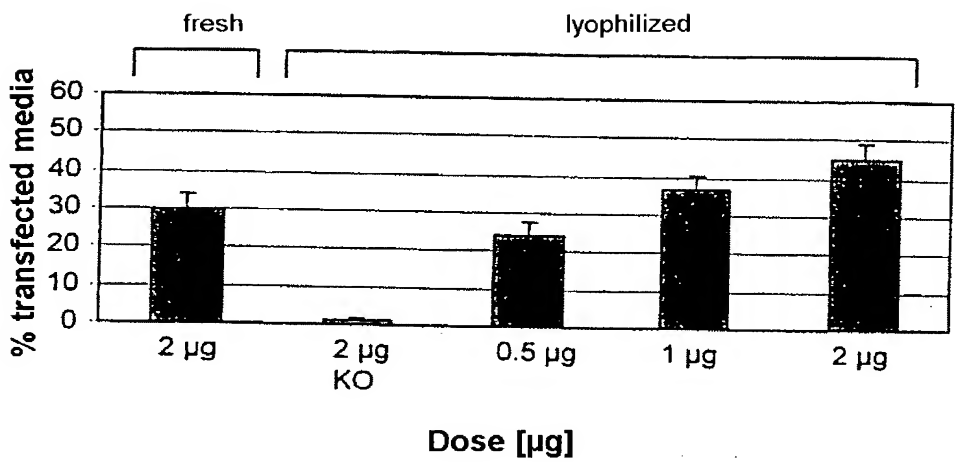

fresh
lyophilized
% transfected media
60
50
40
30
20
10
0
2 µg
2 µg KO
0.5 µg
1 µg
2 µg
Dose [µg]

Fig. 7

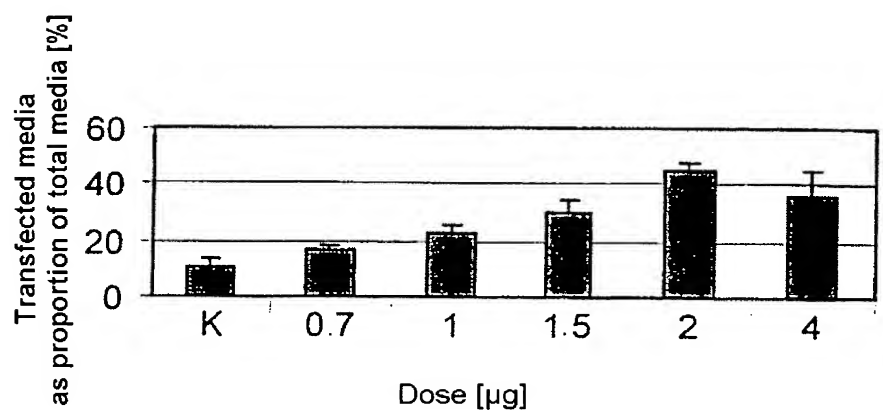

Transfected media
as proportion of total media [%]
60
40
20
0
K
0.7
1
1.5
2
4
Dose [µg]

Fig. 8A

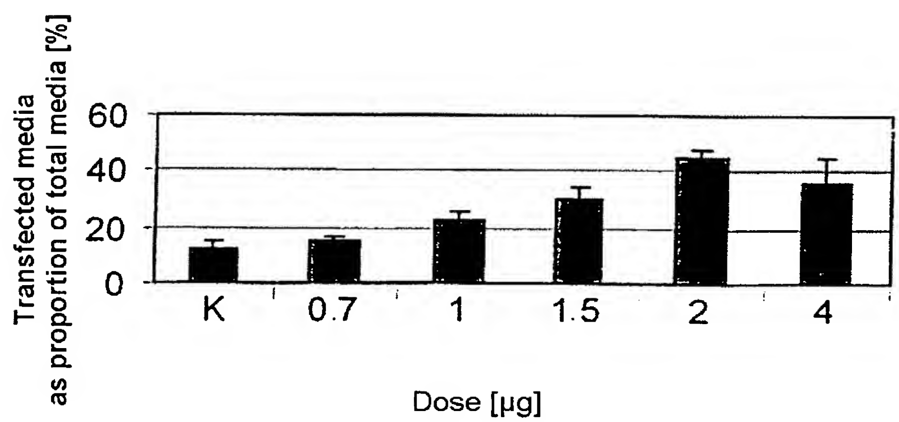

Transfected media
as proportion of total media [%]
60
40
20
0
K
0.7
1
1.5
2
4
Dose [µg]

Fig. 8B

OIPE
JUL 24 2002
PATENT & TRADEMARK OFFICE

10088248.072402

PATENT
ATTORNEY DOCKET NO: 50125/051001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled PHARMACEUTICAL COMPOSITION IN THE FORM OF A NUCLEIC ACID/LIPID COMPLEX, ITS PRODUCTION AND USE IN GENE THERAPY, the specification of which

☐ is attached hereto.
■ was filed on March 15, 2002 as Application Serial No. 10/088,248
and was amended on ____________________.
☐ was described and claimed in PCT International Application No. __________________
filed on ______________ and as amended under PCT Article 19 on _______________.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

FOREIGN PRIORITY RIGHTS: I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
Germany	DE 19944262.2	September 15, 1999	Yes

PROVISIONAL PRIORITY RIGHTS: I hereby claim priority benefits under Title 35, United States Code, § 119(e) and § 120 of any United States provisional patent application(s) listed below filed by an inventor or inventors on the same subject matter as the present application and having a filing date before that of the application(s) of which priority is claimed:

Serial Number	Filing Date	Status

NON-PROVISIONAL PRIORITY RIGHTS: I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by

10088248 .072402

COMBINED DECLARATION AND POWER OF ATTORNEY

the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Filing Date	Status
PCT	PCT/EP00/08996	September 14, 2000

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506, Timothy J. Douros, Reg. No. 41,716, Vicki Healy, Reg. No. 48,343.

Address all telephone calls to: Karen L. Elbing, Ph.D. at 617/428-0200.

Address all correspondence to: Karen L. Elbing, Ph.D. at Clark & Elbing LLP, 101 Federal Street, Boston, MA 02110. **Customer No: 21559**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name (First, Middle, Last)	Residence Address (City, State, Country)	Post Office Address (Street, City, State, Country)	Citizenship
1 - CO Jens Schletter DEX	Hilden, Germany	Lortzingstr. 27 40742 Hilden Germany	German
Signature:			Date: 07 June 02